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REGULATION OF TYPE II RESPONSES IN LUNG FIBROSIS AND SYSTEMIC
AUTOIMMUNITY

A Dissertation Presented By

TIA YAA'ASANTEWA BUMPUS

Submitted to the Faculty of the University of Massachusetts Graduate School of
Biomedical Sciences, Worcester in partial fulfillment of the requirements for the
degree of

DOCTOR OF PHILOSOPHY

APRIL 9, 2014

IMMUNOLOGY

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ABSTRACT

Preclinical models of lupus indicate that T cell-B cell collaboration drives antinuclear antibody (ANA) production and sustains T cell activation. Autoreactive B lymphocytes are present in the normal repertoire but persist as ignorant or anergic cells. Mechanisms that normally limit T cell activation of autoreactive B cells remain incompletely resolved, but potentially include the absence of autoreactive effector T cell subsets and/or the presence of autoAg-specific regulatory T cells (Tregs). Several studies have addressed this issue by using experimental systems dependent on transgenic autoreactive B cells, but much less is known about the activation of autoreactive B cells present in a polyclonal repertoire. In the second chapter of this thesis, I have explored the role of effector T cells and Tregs using mice that express an inducible pseudo-autoAg expressed on B cells and other antigen presenting cells (APCs). In this system, activated Th2 cells, but not naïve T cells, elicit the production of ANAs, but ANA production is severely limited by autoAg-specific Tregs. Bone marrow chimera experiments further demonstrated that this B cell activation is constrained by radioresistant autoantigen-expressing APCs (rAPC) present in the thymus as well as by non-hematopoietic stromal cells located in peripheral lymphoid tissue. Importantly, peripheral rAPC expression of autoAg induced the expansion of a highly effective subset of CD62L⁺CD69⁺ Tregs.

The third chapter of this thesis focuses on the contribution of CD8⁺ T cells to fibrosis resulting from sterile lung injury. Type 2 effector production of IL-13 is

a demonstrated requirement in several models of fibrosis, and is routinely ascribed to CD4⁺ Th2 cells. However, we now demonstrate a major role for pulmonary CD8⁺ T cells, which mediate an exaggerated wound healing response and fibrosis through robust differentiation into IL-13-producing pro-fibrotic type 2 effectors (Tc2). Remarkably, differentiation of these Tc2 cells in the lung requires IL-21. We further show that the combination of IL-4 and IL-21 skews naïve CD8⁺ T cells to produce IL-21, which in turn acts in an autocrine manner to support robust IL-13 production. TGF- β negatively regulates production of IL-13 by suppressing CD8⁺ T cell responsiveness to IL-21. Our data illuminate a novel pathway involved in the onset and regulation of pulmonary fibrosis, and identify Tc2 cells as key mediators of fibrogenesis.

TABLE OF CONTENTS

APPROVAL PAGE	ii
ACKNOWLEDGEMENTS	iii
ABSTRACT	v
TABLE OF CONTENTS	vii
LIST OF FIGURES	viii
LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE	x
ATTRIBUTIONS	xi
CHAPTER I: Introduction	1
CHAPTER II: A specific role for secondary lymphoid organ stroma in the activation and elaboration regulatory T cells to suppress autoantibody production	
<i>Introduction</i>	<i>20</i>
<i>Development of a transgenic mouse expressing an inducible autoAg.....</i>	<i>24</i>
<i>li-TGO mice develop lupus-like autoimmune disease following adoptive transfer of activated DO11.10 T cells.....</i>	<i>27</i>
<i>Non-induced TGO expression by radioresistant APCs drives extensive Treg development.....</i>	<i>33</i>
<i>Absence of self-Ag bearing stromal cells is permissive to persistent autoreactive T cell activation and enhanced autoreactive B cell activity.....</i>	<i>39</i>
<i>Activation and elaboration of Ag-specific SLO-resident Tregs are preferentially activated by rAPCs.....</i>	<i>40</i>
<i>Discussion.....</i>	<i>51</i>
<i>Materials And Methods</i>	<i>56</i>
CHAPTER III: IL-21 Drives Pulmonary Fibrosis through the Induction of Pro- fibrotic CD8⁺ T Cells	
<i>Introduction.....</i>	<i>61</i>
<i>IL-21R signaling drives fibrosis but is dispensable for inflammation after sterile lung injury.....</i>	<i>64</i>
<i>CD8⁺ T cell recruitment is impaired in Il21r^{-/-} mice after sterile lung injury.....</i>	<i>67</i>
<i>Protection from fibrosis in Il21r^{-/-} mice is associated with a decrease in IL-13, but not IL-17A, production by T cells.....</i>	<i>70</i>
<i>IL-21 promotes Tc2 differentiation in vivo and in vitro.....</i>	<i>78</i>
<i>The combination of IL-4 and IL-21 efficiently promotes the in vitro differentiation of CD8⁺ T cells into Tc2²¹ cells.....</i>	<i>81</i>
<i>Tc2 cells self-prime with IL-21.....</i>	<i>86</i>
<i>CD25 signaling and TGF-β regulate Tc2 differentiation.....</i>	<i>89</i>
<i>IL-21R-sufficient CD8⁺ T cells restore fibrotic phenotype of IL-21R-deficient mice</i>	<i>92</i>
<i>Discussion.....</i>	<i>98</i>
<i>Experimental Procedures.....</i>	<i>108</i>
Chapter IV: Discussion.....	115

LIST OF FIGURES

Figure 2.1. Naive autoreactive T cells proliferate robustly in response to self-Ag.	26
Figure 2.2. B cell expression of TGO can drive DO11 proliferation.	30
Figure 2.3. Activated Th2, but not naïve autoreactive T cells can elicit autoantibody production in li-TGO mice.	32
Figure 2.4. Ectopic expression of self-Ag on radioresistant APCs drives extensive Treg development independent of endogenous α -chain expression.....	36
Figure 2.5. Non-induced expression of TGO by radioresistant APCs drives extensive DO11 deletion and proportional Treg increase.	38
Figure 2.6. Absence of self-Ag bearing stromal cells is permissive ANA production by autoreactive B cells without sublethal irradiation.	42
Figure 2.7. Absence of self-Ag bearing stromal cells is permissive to increased B cell activation.	44
Figure 2.8. Self-Ag presentation by radioresistant APCs drives activation and elaboration of SLO-tethered Tregs.	47
Figure 2.9. Cognate Ag presentation by LNSC reinforces Treg capacity for lymph node homing.	50
Figure 3.1. IL-21R deficiency does not protect from bleomycin-induced lung inflammation.....	66
Figure 3.2. IL-21R signaling is required for fibrosis following bleomycin-induced lung inflammation.....	69
Figure 3.3. IL-21/IL-21R interactions are required for optimal CD8 ⁺ T cell recruitment to the lung following bleomycin injury.	72
Figure 3.4. Increased Treg activity is not responsible for protection from fibrosis in IL- 21R deficient mice.	74
Figure 3.5. Lack of IL-21R significantly impairs IL-13 production in response to sterile lung injury.....	77
Figure 3.6. IL-21 is constitutively expressed by a small population of lung CD4 ⁺ T cells, and is upregulated in both CD4 and CD8 T cells in response to bleomycin or IL-21 i.t..	80
Figure 3.7. Sterile lung injury and IL-21 drives Tc2 differentiation in the lung and fibrosis.	83
Figure 3.8. IL-21 drives Tc2 differentiation in vitro.	85
Figure 3.9. Autocrine IL-21 promotes Tc2 phenotype.....	88
Figure 3.10. Tc2 ²¹ cells are profibrotic in vivo.	91
Figure 3.11. CD25 signaling and TGF- β regulate Tc2 differentiation <i>in vitro</i>	94

Figure 3.12. IL-21R on CD8 T cells is required for optimal IL-13 production and collagen deposition.....	97
Figure 3.13. Increased cytotoxicity of CD4+ T cells may promote fibrosis.....	100

LIST OF SYMBOLS, ABBREVIATIONS OR NOMENCLATURE

APC, antigen presenting cell
 α -smAc, α -smooth muscle actin
 Ags, antigens
 BALF, bronchoalveolar lavage fluid
 BCR, B cell receptor
 Bleo, bleomycin
 BM, bone marrow
 CD, cluster of differentiation
 CFSE, carboxyfluorescein succinimidyl ester
 CNS, conserved noncoding sequence
 cTEC, cortical thymic epithelial cell
 DC, dendritic cell
 Dox, doxycycline
 ELISA, enzyme-linked immunosorbent assay
 GFP, green fluorescent protein
 hAPC, hematopoietically-derived antigen presenting cell
 Ig, immunoglobulin
 IL, interleukin
 IPF, idiopathic pulmonary fibrosis
 i.t., intratracheal
 i.v. intravenous
 MHC, major histocompatibility complex
 mTEC, medullary thymic epithelial cell
 OVA, chicken ovalbumin
 pOVA, chicken ovalbumin peptide
 rAPC, radioresistant antigen presenting cell
 rtTA, reverse tetracycline transactivator
 SLE, systemic lupus erythematosus
 SSc, systemic sclerosis
 TCR, T cell receptor
 TGF- β , transforming growth factor- β
 TGO, transmembrane domain of transferrin, **g**reen fluorescent protein, chicken
ovalbumin
 TRE, tetracycline responsive element

ATTRIBUTIONS

Karen S. Lee generated Figures 2.1A and 2.1B, which were published in her dissertation “A Tet-regulatable mouse model for the analysis of systemic autoimmune disease” in 2008 (Boston University School of Medicine).

Patricia Busto performed the ELISAs that generated the data shown in Figure 2.3B.

CHAPTER I

Introduction

Foundations of self/non-self discrimination and immunological tolerance

The immune system must reliably and continually distinguish between self and non-self antigens (Ags) in order to avert autoimmunity and mount appropriate responses to pathogens and foreign proteins. The recognition of non-self by immune cells, leads to the pathophysiology of transplant rejection as well as appropriate immune responses to potentially harmful foreign Ags. Induction of immunological tolerance to self-Ags occurs via complex mechanisms in central lymphoid organs (thymus and bone marrow) and peripheral tissues, resulting in non-reactivity to self-Ags.

In vivo evidence of immunological tolerance was first described by Ray Owen using studies from bovine twins with *in utero* vascular anastomoses, which he described as “nature’s experiment with parabiosis.” In these bovine twins, each co-twin possessed two antigenically distinct populations of red blood cells (RBCs) throughout adulthood. However, the offspring of a twin sire would not inherit both populations of RBCs. Furthermore, twins with different sires also had two blood types, one of which could not have been inherited from either the sire or the dame (Owen, 1945). This evidence suggests that bovine twins exchanged RBC precursors *in utero* that were accepted as “self” by the animal, despite being genetically distinct from all other cells. These studies provided a

foundation for Peter Medawar's remarkable finding that not only monozygotic, but dizygotic twins, could accept skin grafts from a co-twin. Ordinary sibs, however, were not tolerant to skin grafts as in the case of dizygotic twins (Anderson et al., 1951). The bovine twin studies also provided a partial basis for Fenner and Burnet's theory of immune tolerance—namely that exposure to an Ag early in life would lead to tolerance to the same Ag later in life. This theory provided a framework for understanding the active process by which autoimmunity is prevented during a lymphocyte's development and throughout the life of that cell.

Induction of immunological tolerance requires deletion or functional inactivation of self-reactive lymphocytes during development, as well as inactivation during scenarios that could lead to activation of mature autoreactive lymphocytes in tissues. Unique surface receptors, which allow for an adaptive immune response to a vast number of Ags, are generated during development through genetic recombination. This process routinely results in self-reactive Ag receptor expression by T and B lymphocytes. While most autoreactive lymphocytes are deleted before full maturation and emigration from central lymphoid organs, between 5-10% of the peripheral blood T or B cell population is autoreactive in healthy humans, yet only 3-8% of human suffer from autoimmune diseases (Goodnow et al., 1995). A variety of intrinsic and extrinsic factors are responsible for the control of autoreactive lymphocytes after their development and will be discussed separately for T and B lymphocytes.

B cell tolerance

In the bone marrow, pre-B cells undergo somatic rearrangements to generate receptor diversity in a process called V(D)J recombination. Briefly, the variable (V), diversity (D), and joining (J) gene segments are cleaved and recombined with the potential to yield billions of unique receptor specificities. Each B cell generally expresses one receptor (although dual-receptor B cells have been reported (Li et al., 2002)). The randomness of V(D)J recombination, while generating BCRs that could theoretically aid in protection from any pathogen, also leads to the generation of autoreactive BCRs. In fact, it has been estimated that about 75% of early immature B cells and 30% of mature B cells are autoreactive in humans (Wardemann et al., 2003). Strong BCR crosslinking upon binding self-Ag leads to elimination (clonal deletion) (Nemazee and Burki, 1989; Nossal and Pike, 1978) or revision of the BCR (receptor editing) (Radic et al., 1993; Tiegs et al., 1993). Clonal deletion is apoptotic death as the result of a self-reactive B cell binding self-Ag in the bone marrow, and is thought to be the result of binding to multivalent self-Ag. Immature B cells are highly susceptible to deletion upon self-recognition (King and Monroe, 2000). Receptor editing can occur when the BCR binds self-Ag, triggering upregulation of RAG genes, which leads to further rearrangement of Ig light chain genes, thus pairing the heavy chain with a new light chain (Goodnow et al., 1995). However, the processes of clonal deletion and receptor editing are incomplete in eliminating autoreactive B cells from the B cell repertoire in the bone marrow, and autoreactive cells can

exit the bone marrow (Wardemann et al., 2003). In both humans and mouse models, self-recognition by mature B cells can lead to anergy (Goodnow et al., 1988; Zikherman et al., 2012). Anergic cells are not stimulated under conditions in which a normal mature B lymphocyte would become activated (for example, cognate Ag or anti-IgM) and have a significantly shorter half-life than normal mature lymphocytes. Autoreactive mature B cells in the periphery can also be ignorant of self-Ag, meaning that effective activation by self-Ag is unavailable to the B cell for reason that include very low concentration of Ag and Ags present only in areas from which B cells are normally excluded.

The foregoing discussion describes B cell tolerance as largely intrinsic. It is important to note here that T cell-B cell interactions can have significant influence on the “re-awakening” of an anergic autoreactive B cell (Seo et al., 2002), but can also eliminate anergic B cells (Rathmell et al., 1995).

Autoreactive B cells have been most intensively studied in the prototypic autoimmune disease systemic lupus erythematosus (SLE), where B cells invariably produce (potentially pathogenic) autoantibodies. However, B cells are important effectors in autoimmunity—even in organ-specific diseases where T cells are thought to be the major force in disease pathogenesis—through Ag-presentation, cytokine production, and antibody production (Barr et al., 2012; Diana et al., 2013; Falcone et al., 1998; Lyons et al., 1999; Marino et al., 2012; Matsushita et al., 2008; Pierson et al., 2014; Silveira et al., 2002). In chapter II, I will describe my results from a model system that studies the result of cognate

interactions between T cells specific for a neo-self Ag and naturally occurring autoreactive B cells.

T cell development and central tolerance

The importance of the thymus in immune tolerance was demonstrated by experimental neonatal thymectomy in mice, which can result in multiorgan autoimmune disease (with predilection gonads and endocrine organs) (Kojima and Prehn, 1981; Nishizuka and Sakakura, 1969). As the T cell receptor (TCR) is also generated through somatic rearrangements, autoreactive TCRs are expressed by a substantial frequency of developing thymocytes. $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells and $CD4^-CD8^-$ $\gamma\delta$ T cells develop in the thymus from bone marrow-derived lymphoid precursors. The outer layer of the thymus (cortex) and inner region (medulla) contain large numbers of thymocytes (developing T cells). The cortex contains both CD4 and CD8 double-negative (DN) and CD4 and CD8 double positive (DP) thymocytes. The medulla contains CD4 or CD8 single-positive (SP) thymocytes. Both the cortex and the medulla also contain thymic epithelial cells (cTECs and mTECs, respectively) and thymic dendritic cells (DCs), which serve an important function in both T cell development and T cell tolerance. Upon migration to the thymus, lymphoid progenitors commit to the T cell lineage following interactions with thymic stroma. In addition to chemokines that guide thymocyte migration, cTECs express IL-7 and Notch ligands to support T cell development. In the cortex, DN thymocytes undergo proliferation and

TCR gene rearrangements. At the DN2 stage, thymocytes express CD25 (the high-affinity IL-2 receptor; IL-2R α) and rearrangement of the β chain (or γ and δ for progenitors of $\gamma\delta$ T cells) begins, which continues and increases during DN3. At this stage, a surrogate pre-TCR- α chain is expressed. Signaling through the newly formed pre-TCR halts β -chain rearrangements. During the DN4 stage, thymocytes proliferate extensively. DP thymocytes re-express RAG genes to undergo α -chain rearrangements. The majority of $\alpha\beta$ pairings will result in nonproductive TCRs. Recognition of self-peptide:self-MHC complexes on cTECs and DCs promote progression to the SP stage and survival. The majority of thymocytes do not receive a rescue signal as a result of this interaction and die by apoptosis, termed death by neglect (Germain, 2002; Rothenberg et al., 2008).

Strong TCR-self-Ag binding (occurring in about 5% of developing T cells (Goodnow et al., 2005)) can lead to clonal deletion of self-reactive T cells in the thymus, a mechanism of autoimmunity prevention. The role of the cortex in negative selection has been debated, but can occur (Kisielow et al., 1988). The medulla appears to be the primary site for negative selection. Cortical SP thymocytes migrate to the medulla via CCR7-mediated chemotaxis toward ligands expressed by mTECs (Forster et al., 2008; Misslitz et al., 2004; Takahama, 2006). mTECs are crucial for both deletion of thymocytes that have high-avidity interactions with self-peptide:MHC complexes, and regulatory T cell (Treg) development. The transcription factor AIRE (autoimmune regulator) is expressed by mTECs and controls ectopic, thymic expression of a number of

tissue specific Ags (TSA) (Anderson and Su, 2011; Anderson et al., 2002) that could become auto-Ags without tolerization of the T cell repertoire. Deficiency in AIRE causes severe autoimmunity in humans and mice, and is due, at least in part, to escape of strongly autoreactive T cells from negative selection. In humans, this disease, autoimmune polyendocrine-candidiasis-ectodermal dystrophy (APECED), is characterized by damage to cells expressing TSAs (such as those expressed in endocrine organs) that are not normally expressed in the thymus (Anderson and Su, 2011; Xing and Hogquist, 2012). To avoid the persistence of T cell clones with autoreactivity to TSAs, AIRE-driven expression and subsequent negative selection, or diversion of TSA-specific cells into a regulatory cell type, is required. In addition to self-Ag presentation by mTECs, DCs are abundant in the medulla relative to the cortex, and can present mTEC-derived self-Ag (Gallegos and Bevan, 2004) and Ags from the blood on MHC (Xing and Hogquist, 2012).

T cell peripheral tolerance

T cell central tolerance, while effective, is incomplete, and not all Ags that can be encountered in the periphery are expressed in the thymus. Autoreactive T cells that are activated by self-Ag in peripheral tissues can be eliminated by deletion or anergy, and it is likely that absence of tolerance mechanisms such as these could cause autoimmunity. Several checkpoints exist to ensure that appropriately activated T cells survive, and aberrantly activated T cells die. In

addition to TCR ligation, simultaneous costimulatory signaling through CD28 is also required, which usually comes from CD80 and CD86 on APCs. Activation of APCs through innate immune receptors by microbial products causes upregulation of costimulatory molecules, ensuring that T cells become activated under appropriate circumstances. TCR ligation in the presence of costimulation can drive the secretion of paracrine and autocrine growth factors, such as IL-2, as well as proliferation and differentiation into effector cells. Anergy is a possible outcome of self-Ag recognition by self-reactive T cell, namely when the T cell repertoire is chronically exposed to an Ag (such as a ubiquitous self-Ag) or when Ag is presented under sub-immunogenic circumstances, such as the absence of costimulation (Josefowicz et al., 2012; Schwartz, 2003; Xing and Hogquist, 2012). Activated autoreactive T cells express death receptors such as Fas and can be eliminated by fratricidal T cells bearing the appropriate ligand. This class of programmed cell death, termed activation-induced cell death (AICD), can occur after TCR ligation (Ju et al., 1995), or after repeated antigen stimulation (Kawabe and Ochi, 1991). Mice that are deficient in Fas or Fas ligand (FasL) (mouse strains *lpr* and *gld*, respectively, develop severe lymphoproliferative disease and systemic autoimmunity (Cohen and Eisenberg, 1991).

Deletional tolerance to TSAs can occur in peripheral tissues. As in the thymus, TSAs can be ectopically expressed and presented in secondary lymphoid organs (SLO) such as the spleen and lymph nodes. Deaf1 is a transcriptional regulator expressed by peripheral lymph node stromal cells

(LNSC), and drives transcription of TSAs (Fletcher et al., 2010; Yip et al., 2009), which could lead to deletional tolerance. AIRE is also expressed by APCs in peripheral tissue, but mediates transcription of genes that are distinct from AIRE-dependent genes expressed by mTECs (Gardner et al., 2008). Chapter II of this thesis addresses the role of LNSC in peripheral tolerance by modulation of Treg activity.

Treg development and role in peripheral tolerance

Despite extensive recessive tolerance in developing and mature T cells, immunoregulation is required during homeostasis and during appropriate immune responses. Dominant tolerance – the active regulation of immune responses by Tregs – is established in the thymus during T cell development and reinforces the inhibition of autoreactive or over-exuberant immune responses. Conventional Tregs are CD4⁺ T cells that express both CD25 and the transcription factor Foxp3 – the master regulator of Treg function (Fontenot et al., 2003; Hori et al., 2003). Deficiency of Foxp3 causes IPEX syndrome (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) in humans and mice, a fatal, T cell-driven autoimmune disease. A prevailing model of Treg development proposes a TCR driven (instructive) component as well as secondary and tertiary factors such as cytokine signaling and costimulation (Josefowicz et al., 2012). The instructive component of the model posits intermediate avidity, rather than low avidity (as is required for positive selection)

or high avidity (autoreactive) self-peptide:self-MHC interactions with TCRs drives Treg development (Josefowicz et al., 2012; Lee et al., 2012; Xing and Hogquist, 2012). In addition to self-specificity, IL-2 signaling through CD25 is required for Treg maintenance and optimal Treg development (Fontenot et al., 2005). The role of IL-2 in tolerance was described by deletion of IL-2 or IL-2R α (CD25), which led to severe autoimmunity and T cell deregulation (Sadlack et al., 1995; Sadlack et al., 1993; Willerford et al., 1995). Other common gamma chain cytokines such as IL-7 and IL-15 may compensate for loss of IL-2 signaling in developing Tregs (all signal through STAT5) (Vang et al., 2008). Mice with T cell specific ablation of STAT5 also exhibit a more severe reduction in Tregs (Burchill et al., 2007).

TGF- β has an important role in extrathymic Treg development (Bilate and Lafaille, 2012), however, the exact role of TGF- β in thymic Treg development is less clear. Mice with T cell lineage-specific deletion of TGF- β RI develop fewer Tregs during the first several days after birth; however, an IL-2 dependent compensatory increase follows later (Li et al., 2006; Liu et al., 2008). TGF- β RII-deficient mice have increased thymocyte death in response to anti-CD3 injections. Moreover, mice expressing chicken ovalbumin (ova) in pancreatic islets crossed to OT-II (ova-specific transgenic CD4⁺ T cells) exhibited increased negative selection and decreased thymic Tregs in the absence of TGF- β RII. This reduction in thymic Tregs in TGF- β RII deficient mice is due to Bim mediated apoptosis (Ouyang et al., 2010). These data implicate TGF- β signaling as a

critical positive regulator of thymic Tregs through promoting survival during negative selection. The transcription factor Smad3 is downstream of TGF- β signaling and can bind a *Foxp3* enhancer element (Tone et al., 2008). This enhancer element, CNS1, appears to be dispensable for thymic Treg generation, but required for peripheral Treg development (Zheng et al., 2010); however, this has been disputed (Schlenger et al., 2012).

In peripheral tissues, CD4⁺ T cells specific for foreign (such as environmental or food Ags) and self-Ags (such as those derived from apoptotic debris) can differentiate into Tregs (induced Tregs; iTregs). For example, apoptotic debris uptake by immature (non-activated) APCs can induce differentiation of IL-10 producing self-Ag specific Treg (Lutz and Schuler, 2002) and local increases in TGF- β that can drive *Foxp3* expression (Perruche et al., 2006). Higher affinity TCR interactions may also determine Treg versus non-Treg fate peripherally (Gottschalk et al., 2010), and TCR specificity appears to be key, although there is only partial overlap between TCR repertoires of thymic Tregs and peripherally induced Tregs (Lathrop et al., 2008). Treg peripheral induction can also occur in response to chronic exposure to Ag (Daniel et al., 2011; Kretschmer et al., 2005), suggesting that there may be cell-intrinsic qualities that dictate the fates of anergy versus iTreg.

Tregs restrain both appropriate immune responses and immune responses against self-Ags. CD4⁺CD25⁺*Foxp3*⁺ Tregs suppress immune responses using both contact-dependent, and secreted factors (Feuerer et al.,

2009; Shevach, 2009). Tregs can modulate local levels of T cell nutrients and growth factors to limit effector T cell function. For example, high levels of CD25 expression on Tregs leads to high IL-2 consumption, and decreases IL-2 availability for activated lymphocytes (Gasteiger et al., 2013; Pandiyan et al., 2007; Sitrin et al., 2013). CTLA-4 is also crucial for Treg-mediated suppression, and Treg-specific CTLA-4 deficiency leads to lymphoproliferation and autoimmunity (Wing et al., 2008), and sustained CTLA-4 expression by Tregs mediates protection from the autoinflammatory disease that occurs in *Ctla4*^{-/-} mice (Friedline et al., 2009).

Foxp3 expression has been reported in other regulatory T cell types, including CD8⁺ T cells (Robb et al., 2012; Suzuki et al., 2012) and T follicular helper cells (Tfh) (Chung et al., 2011; Linterman et al., 2011; Wollenberg et al., 2011), but the contribution of these unconventional Foxp3⁺ cells has not been fully elucidated.

Tregs can respond to the inflammatory milieu generated by Th1, Th2, and Th17 cells (described in the following section), modifying Treg trafficking and suppression modalities. Treg-specific deletion of STAT3 leads to uncontrolled Th17 responses, which is accompanied by decreased expression of IL-6R, IL-1 β R, and CCR6. CCR6 promotes Th17 recruitment to sites of inflammation (Esplugues et al., 2011; Hirota et al., 2007; Wang et al., 2009), and thus could promote Treg recruitment to sites of Th17 inflammation to restrain inflammation. Similarly, Th2 development is dependent on IRF4 (Lohoff et al., 2002;

Rengarajan et al., 2002), and expression of IRF4 is required to suppress Th2 responses (Zheng et al., 2009). However, IRF4 may also be important in the differentiation of Th17 cells (Brustle et al., 2007). Transcription factors discovered in the context of a particular Th subset can be important for the development of more than one type of Th (for example, IRF4 (Brustle et al., 2007) or STAT3 (Stritesky et al., 2011)). Thus, the requirement for Treg expression of specific transcription factors to suppress specific Th responses is likely more complex than programming Tregs to become suppressive homologues of Th subsets.

Effector T cell differentiation

The research chapters of this thesis will present data on preclinical models of autoimmune and autoinflammatory diseases that are in part dependent on aberrantly activated T and B lymphocytes. T cells mediate autoimmunity through providing help to B cells, secreting cytokines that promote inflammation, and causing tissue damage. Recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by the innate immune system triggers T cell responses that are tailored to different pathogen types. The resulting cytokine milieu is a key factor in determining T cell differentiation, which is largely governed by transcription factors downstream of cytokine receptors. T cell subsets have been largely defined by production of a few key cytokines at the exclusion of production of other cytokines, however, T

cell subsets can exist along a continuum. As these canonical subsets have quite different effector functions in both appropriate immune responses and in autoimmunity, a brief discussion of T cell differentiation and function is apropos.

Th1 and Tc1 cells are CD4⁺ or CD8⁺ T cells, respectively, which secrete IFN- γ . T-bet is the master regulator of Th1 differentiation, and IFN- γ is a major inducer of T-bet (Lighvani et al., 2001), thus Th1 development is positively regulated by IFN- γ . Required for effective viral clearance, IFN- γ is the “default” cytokine secreted after T cell activation. IL-12 secreted by DCs and macrophages during Ag presentation drives Th1 differentiation (Heufler et al., 1996; Hsieh et al., 1993; Manetti et al., 1993).

CD4⁺ T cells that produce IL-17A (Th17) are implicated in several autoinflammatory conditions. Th17 cells can also produce IL-21, a highly pleiotropic cytokine, and IL-22, which has important functions in barrier immunity (Korn et al., 2009). A combination of IL-6 (or IL-1 β or IL-21) and (low-concentration) TGF- β promote IL-17A production by T cells *in vitro* and *in vivo* and expression of ROR γ T (or human homolog Rorc), a transcription factor that is crucial for Th17 differentiation (Ivanov et al., 2006; Korn et al., 2009). IL-23, produced by macrophages and DCs supports Th17 differentiation and effector cytokine production (Korn et al., 2009; Langrish et al., 2005). IRF4, BATF, and STAT3 are also important regulators of Th17 differentiation (Littman and Rudensky, 2010). A subset of CD8⁺ T cells also produces IL-17A (Tc17), and can be differentiated *in vitro* under Th17-skewing conditions (Hamada et al.,

2009; Yen et al., 2009). *In vivo*, Tc17 cells are protective following vaccination (Nanjappa et al., 2012) and viral infection (Hamada et al., 2009; Yeh et al., 2010).

Th2 cells provide protection from helminth infection, are elicited by allergens and cause symptoms of allergy, and are involved in both normal and pathological tissue repair (fibrosis). Th2 cells are at the helm of type 2 responses, defined by production of IL-4, IL-5, and IL-13 and recruitment of basophils, eosinophils, and mast cells (Oliphant et al., 2011). IL-4 drives Th2 differentiation (Swain et al., 1990), and IL-2 is also important (Ben-Sasson et al., 1990; Le Gros et al., 1990). Th2 responses can also be initiated by the release of innate-derived factors such as thymic stromal lymphopoietin (TSLP) (Paul, 2010), IL-25 and IL-33 (Oliphant et al., 2011), the latter of which can be released by damaged cells (Moussion et al., 2008). Type 2 responses drive normal tissue repair, as well as unchecked operation of wound healing pathways (fibrosis) (Wynn, 2004).

Th2/Tc2 cells and fibrosis

Repair processes that are activated by wounding attempt to restore normal tissue architecture. An initial inflammatory response consisting of innate immune cells (mostly neutrophils, monocytes, and macrophages) serves to respond to potential pathogens and close the wound to prevent infection. T cells make up a large percentage of leukocytes at sites of repair during the tissue-remodeling

phase. The extent and type of inflammation impacts the quality of damage repair (Eming et al., 2007). Th1 cells antagonize wound repair pathways (Ishida et al., 2004), in part by negatively regulating fibroblast activation, which is required for production of collagen and tissue repair (Wynn, 2004). Th2 cytokines promote fibroblast activation and the differentiation of pro-repair M2 macrophages (Wick et al., 2013). After injury, macrophages secrete copious TGF- β in response to IL-13 (Lee et al., 2001; Wynn, 2004). Activation of fibroblasts by TGF- β and consequent collagen production is crucial for both wound healing and fibrosis (Wynn and Ramalingam, 2012). Sustained activity of activated fibroblasts results in fibrosis, a deleterious accumulation of collagen and extracellular matrix components that causes organ dysfunction.

Fibrosis is a frequent result of chronic inflammatory conditions, and despite the prevalence of fibrosis as both a primary disease and a consequence of common diseases such as asthma and heart failure, treatments that specifically target fibrogenesis are extremely limited (Wynn and Ramalingam, 2012). Idiopathic pulmonary fibrosis is a chronic debilitating form of lung fibrosis with a high rate of mortality (King Jr et al., 2014). Interstitial lung disease in systemic sclerosis (SSc) is a chronic, progressive fibrosis that is the leading cause of mortality in patients with diffuse disease (Wells et al., 2009). Th2 cytokines such as IL-4 and IL-13 are pro-fibrotic promoting and are elevated in IPF (Murray et al., 2013) and SSc (Hasegawa et al., 1997). Nevertheless, the actual role of activated Tc2 cells relative to Th2 cells and the genesis of Tc2 cells

in vivo is poorly understood, despite evidence that both Tc2 and Th2 persist in patients with fibrosis (Atamas et al., 1999; Fuschiotti et al., 2013; Fuschiotti et al., 2009). Chapter III focuses on a fibrotic response due to sterile tissue injury, and specifically addresses the role of IL-21 in regulating over-exuberant type 2 responses.

IL-21 can regulate T cell differentiation and function

IL-21 is a relatively newly identified common γ -chain cytokine produced mainly by activated CD4⁺ T cells, although it may be produced by NKT cells, $\gamma\delta$ T cells, and CD8⁺ T cells. IL-21 secreted by CD4⁺ T cells promotes CD8⁺ T cell-mediated control of chronic viral infection (Frohlich et al., 2009; Yi et al., 2009), which implicated IL-21 as a form of CD4 help to CD8 cells. IL-21 can promote survival of naïve T cells, and in conjunction with other common γ -chain cytokines (IL-7, IL-15), promotes T cell proliferation (Zeng et al., 2005). IL-21 was previously thought to be a Th2 specific cytokine (Frohlich et al., 2007; Wurster et al., 2002), but more recent data show that IL-21 can be produced by several T cell subsets and can act in an autocrine and paracrine manner (Eto et al., 2011; Korn et al., 2007). The IL-21 receptor is a homodimer consisting of the IL-21R subunit and the common cytokine receptor γ -chain. As with all common γ -chain cytokines, IL-21R binding activates Janus kinases JAK1 and JAK3. The main signal transducer and activator (STAT) that IL-21 activates is STAT3, but can also signal through STAT1, STAT5a, and STAT5b (Rochman et al., 2009).

In addition to the role of IL-21 in promoting T cell responses (Elsaesser et al., 2009; Kasaian et al., 2002; Spolski and Leonard, 2008; Yi et al., 2009), IL-21 is important in both germinal center (Eto et al., 2011; Zotos et al., 2010) and extrafollicular (Lee et al., 2011; Odegard et al., 2008) responses in the generation of antibody (Ozaki et al., 2002). IL-21 is associated with inflammation and autoimmunity, and has pathogenic roles in murine models of type 1 diabetes (Ramanathan et al., 2011; Spolski et al., 2008; Sutherland et al., 2009) and SLE (Bubier et al., 2009; Herber et al., 2007; McPhee et al., 2013; Yang et al., 2013).

T and B cells in SLE

SLE is a progressive, multisystem autoimmune disorder characterized by sustained activation of the innate and adaptive immune systems leading to end-organ damage. Progressive renal disease is a common cause of morbidity and mortality. SLE patients invariably test positive for anti-nuclear antibodies (ANAs), which are common among autoimmune diseases (Marshak-Rothstein, 2006; Rahman and Isenberg, 2008). Disruption of autoantibody (autoAb) production is a promising therapeutic target since the kidney manifestation of SLE, glomerulonephritis, is caused in large part by deposition of immune complexes containing autoAbs and their cognate antigens. The majority of autoAbs generated by SLE patients are specific for macromolecular complexes containing nucleic acids or are specific for nucleic acids themselves (Marshak-Rothstein, 2006). TLR7, which binds single-stranded RNA, and TLR9, which binds DNA,

are required for production of RNA-associated autoAb and DNA associated autoAb, respectively, in murine models of lupus (Christensen et al., 2006). B cells and plasmacytoid dendritic cells can become activated by DNA or RNA-associated protein autoAgs containing endogenous TLR7 and TLR9 ligands, thus further perpetuating T and B cell responses to those targets (Marshak-Rothstein, 2006).

The pathogenic potential for B cells in SLE is not limited to autoantibody production. Following activation, B cells can also produce cytokines that may drive inflammation (Lund, 2008; Wojciechowski et al., 2009) and can present autoAg to T cells (Chan and Shlomchik, 1998; Mamula et al., 1994). B cell deficiency significantly reduced the number of spontaneously activated T cells in a murine model of lupus (Chan and Shlomchik, 1998) and also reduced T cell infiltration into the kidneys independently of antibody secretion (Chan et al., 1999). These studies suggest that B cells may propagate SLE through serving as APCs to autoreactive T cells. These studies implicate a chronic positive feedback loop resulting from interactions between autoreactive T and B cells, which is likely to promote SLE. In chapter II, I examine the activation of endogenous autoreactive B cells by activated T cells, and the tolerance checkpoints that limit autoAb production.

CHAPTER II

A specific role for secondary lymphoid organ stroma in the activation and elaboration regulatory T cells to suppress autoantibody production

Introduction

Systemic lupus erythematosus (SLE) is a progressive, multisystem autoimmune disease characterized by anti-nuclear autoantibody (ANA) production by aberrantly activated autoreactive B cells, immune complex deposition in the kidneys, joints, vasculature and skin, elevated inflammatory cytokine levels in the serum, and persistent autoreactive T cell activity. T and B cells are thought to have a cooperative relationship in SLE, as B cell deficiency significantly reduces the number of T cells exhibiting an activated/memory phenotype and subsequent T cell infiltration of the kidneys in autoimmune prone mice (Chan and Shlomchik, 1998; Chan et al., 1999; Shlomchik et al., 1994). Moreover B cell depletion therapy, including the use of rituximab (an anti-CD20 antibody) can lead to the loss of activated T cells in lupus patients and decreased ANA titers (Furtado and Isenberg, 2013; Sfikakis et al., 2005). Consequently, the biology of autoreactive B cells is an area of active investigation.

Autoreactive B cells with a hyporesponsive phenotype and a decreased half-life (anergy) or that are ignorant of cognate self-Ag exist in healthy humans and mice. Although anergic autoreactive B cells are functionally inactivated, they could theoretically be activated when provided with T cell help. Several studies

have addressed this issue using transgenic B cells. Anti-hen egg lysozyme (HEL) B cells can be activated by soluble self-Ag and T cell help. HEL B cells are rendered anergic in mice that also express soluble HEL and are not activated by soluble Ag even in the presence of T cell help (Cooke et al., 1994).

Conversely, anti-DNA B cells (3H9) are also anergic, but can be re-activated by provision of cognate interactions with T cells (Seo et al., 2002). Prior studies also point to a role for autoreactive T cells in the activation of ignorant B cells (Sweet et al., 2011). These results indicate that the nature of self-Ag and the level of BCR crosslinking influence the activation of autoreactive B cells in response to T cell help, and as such, this question warrants study of endogenous autoreactive B cells.

The contribution of effector T cells to autoreactive B cell antibody secretion and accumulation has been studied *in vivo* by depletion of CD4⁺ T cells in autoimmune prone mice (Santoro et al., 1988; Wofsy, 1986; Wofsy and Seaman, 1985, 1987) and genetic deficiency of key T cell cytokines such as IL-4 and IFN- γ (Nakajima et al., 1997; Peng et al., 1997). In most murine models, Th1 cells, which produce IFN- γ and IL-2, have been identified as pathogenic (Peng et al., 2002), and a Th1 signature has been identified in SLE patients (Theofilopoulos et al., 2001). Th2 cells, are effective B-helper cells at least in part through production of IL-4 and have been largely understudied in the context of lupus despite the fact that IgE autoantibodies—a Th2-dependent isotype—are

increased in patients with active SLE (Charles et al., 2010). Thus, the contribution on Th2 cells to SLE warrants further investigation.

Activation of both antigen presenting cells (APCs) and autoreactive lymphocytes can be restrained by regulatory T cells (Tregs). While many autoimmune diseases appear to be multifactorial, in light of recent evidence of Treg insufficiency, deregulation, and dysfunction in autoimmunity (Buckner, 2010; Long and Buckner, 2011; Yan et al., 2008), determining the role of antigen (Ag)-specific Tregs in engaging self-Ag presenting, autoreactive B cells and tuning their function becomes clinically relevant. Treg Ag-specificity may not be crucial, since several studies have demonstrated that Ag-specific Tregs can prevent the activation of neighboring T cells with different specificities (Tarbell et al., 2004; Thornton and Shevach, 1998). At least two studies have highlighted the requirement for Ag-specific interactions between Tregs and autoreactive B cells for effective suppression of autoantibody production in the context of immunization against tissue-associated Ags (Gotot et al., 2012; Ludwig-Portugall et al., 2009).

Treg development in the thymus depends in part on non-hematopoietically derived radioresistant cells in the thymus, namely medullary and cortical thymic epithelial cells (mTECs and cTECs). mTECs present self-peptide:MHC-II complexes directly, and mTEC-derived self-Ag is presented by neighboring dendritic cells (DCs) (Gallegos and Bevan, 2004; Roman et al., 2010). As in the thymus, non-hematopoietic APCs in secondary lymphoid organs (SLO) can

express transcription factors such as AIRE and Deaf1, which drive ectopic expression of tissue specific Ags. These lymph node stromal cells (LNSC) have an important role in the prevention of autoimmunity by both constitutively expressing and presenting tissue specific Ags and decreasing T cell proliferation. Specifically, LNSC mediate CD8⁺ T cell tolerance through the deletion of autoreactive CD8⁺ T cells and iNOS-dependent suppression of proliferation (Fletcher et al., 2010; Fletcher et al., 2011; Khan et al., 2011; Lukacs-Kornek et al., 2011; Siegert et al., 2011). The role of LNSC in regulating CD4⁺ T cell tolerance—especially in relation to Tregs—is less clear, however, LNSC do constitutively express low levels of MHC-II and upregulate MHC-II in response to inflammatory stimuli (Malhotra et al., 2012).

We have developed a model to study cognate interactions between autoreactive T cells and self-Ag presenting B cells in the presence or absence of Ag-specific Tregs. In this chapter, I show that T cell activation is an important checkpoint in the prevention of autoantibody production, autoreactive T cell activation is sufficient for autoantibody production by autoreactive B cells, and that stromal self-Ag presentation is required to most effectively control autoantibody responses.

Results

Development of a transgenic mouse expressing an inducible autoAg.

In order to study autoreactive B cell responses to cognate help from activated autoreactive T cells, double transgenic mice were generated to express an inducible, systemic pseudo-autoAg. The **TGO** fusion construct was developed to encode the transmembrane domain of transferrin (to target protein expression to the cell surface), green fluorescent protein (for identifying expression of the construct), and chicken ovalbumin (the selected “pseudo-autoAg” that elicits T cell activation)(Saff et al., 2004). This construct was cloned into a tetracycline-responsive element (TRE)-containing plasmid and used to generate the TRE-TGO mouse. The reverse tetracycline transactivator (rtTA) activates expression of TGO in response to tetracycline or the synthetic derivative doxycycline (dox) (Rosenblum et al., 2011), although expression levels are sufficiently low to preclude detection of GFP. When TRE-TGO is crossed to the li-rtTA transgenic line, expression of OVA in MHC class II⁺ cells can be induced by dox (Figure 2.1A). This cross, referred to as li-TGO, enables TGO to serve as a pseudo-autoAg where OVA is expressed by all MHC-II⁺ cells and is continually presented to autoreactive CD4⁺ T cells for as long as the mice are maintained on dox. To confirm the inducible expression of the TGO construct and effective presentation on MHC-II, we adoptively transferred naïve CFSE-labeled OVA-specific DO11.10 (DO11) CD4⁺ T cells into li-TGO mice in the presence or absence of dox. DO11 cells divide in li-TGO mice only in the presence of dox (Figure 2.1B),

Figure 2.1. Naïve autoreactive T cells proliferate robustly in response to self-Ag.

(A) Schematic of the TRE-TGO construct and breeding strategy for generating li-TGO mice.

(B) 5×10^6 magnetically purified splenic CD4⁺ T cells from naïve DO11 mice were CFSE labeled and then adoptively transferred into li-TGO mice consuming normal chow or dox chow. At day 5 post-injection, spleens were harvested from mice and analyzed by flow cytometry.

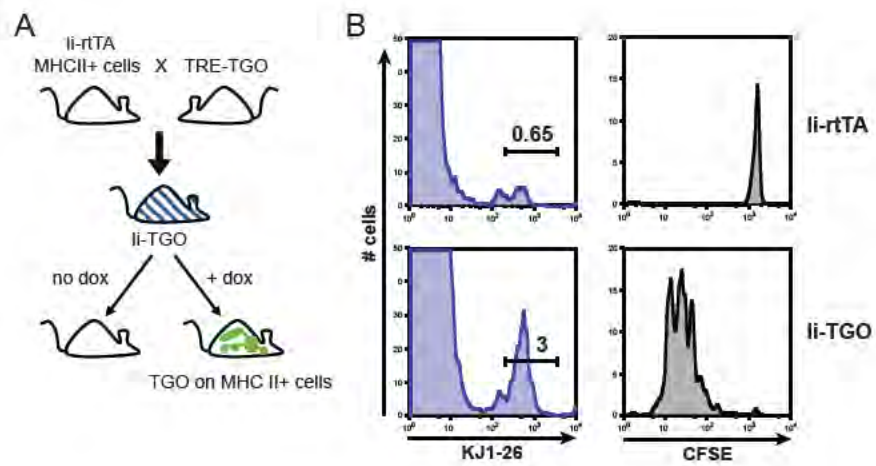


Figure 2.1. Naive autoreactive T cells proliferate robustly in response to self-Ag.

demonstrating that TGO expression and presentation by APCs is sufficient to induce Ag-specific T cell division and is tightly controlled by dox administration.

li-TGO mice develop lupus-like autoimmune disease following adoptive transfer of activated DO11.10 T cells.

Chronic amplification loops due to sustained T cell-B cell interaction are thought to promote systemic autoimmunity in B cell driven diseases such as lupus. For autoreactive B cells activated by autoAg complexes that incorporate TLR ligands, unprimed T cells have clearly been shown to enhance the extent of autoantibody production and isotype switching of transgenic B cells (Seo et al., 2002; Sweet et al., 2011). We sought to determine whether autoreactive T cells specific for a systemically expressed neo-self-Ag could elicit autoantibody production from a naïve normal B cell repertoire. To address this question, we first adoptively transferred either naïve DO11 cells or *in vitro* activated Th2 DO11 T cells into li-TGO mice fed normal or doxycycline chow. Over a period of up to 12 weeks, neither the naïve DO11 or activated T cells induced ANA production, suggesting that cognate interactions between naïve T cells and unprimed autoreactive B cells was not sufficient for autoantibody production. Previous work from our lab has shown that activated but not unprimed B cells express detectable levels on TGO mRNA in the presence of dox. To determine whether naïve DO11 T cells could engage li-TGO B cells *in vivo*, we purified li-TGO B cells and pulsed them with ova peptide (pOVA), dox, or PBS (diluent) *in vitro*. We then adoptively

transferred CD4⁺ T cells purified from DO11 *Rag2*^{-/-} mice and li-TGO B cells into *Rag2*^{-/-} mice on normal or dox chow. Splenocytes from these mice were analyzed after 5 days by flow cytometry. Adoptive transfer of li-TGO B cells into *Rag2*^{-/-} mice on dox promoted DO11 T cell division, but not to the extent that pOVA pulsed B cells promoted T cell division (Figure 2.2). The DO11 cell division could have resulted from a host APC presenting OVA that was acquired from the adoptively transferred T cells, however, this result did confirm production of TGO by B cells.

Sub-lethal irradiation eliminates large numbers of mature T and B cells, and could provide “space” for adoptively transferred T cells. We skewed DO11 Th2 cells *in vitro* which made IL-4 and IL-13, but not IFN- γ or IL-17A (Figure 2.3A) Groups of dox-fed recipient mice were irradiated (400R) and then injected with naïve or activated DO11 Th2 cells. Only a small percentage of adoptively transferred DO11 Th2 cells were recovered from the spleen at 6 weeks (Figure 2.3B). As early as 6 weeks after Th2 transfer, li-irradiated TGO mice produced autoantibodies with a predominantly antinuclear staining pattern (Figure 2.3C). Moreover, li-TGO mice receiving activated self-Ag-specific T cells also showed evidence of IgG and complement deposition in the kidney (Karen Lee’s thesis; data not shown). Importantly, when comparing total Ig isotype titers of the ANA+ recipients, IgG2a and IgG2b only increased 2 fold (Figure 2.3D), suggesting that autoreactive B cells were preferentially activated by the DO11 Th2 cells, compared to other (non-autoreactive) B cells in the peripheral pool. These

Figure 2.2. B cell expression of TGO can drive DO11 proliferation.

5×10^6 CD4⁺ T cells from naïve DO11 mice and 5×10^6 li-TGO B cells were CFSE labeled and then adoptively transferred into li-TGO mice consuming normal chow or dox chow. Prior to adoptive transfer, one set of B cells was pulsed with pOVA. At day 5 post-injection, spleens were harvested from mice and analyzed by flow cytometry for CFSE dilutions, CD4 and B220, to detect CD4⁺ T cells and B cells, respectively. n=3-5 mice per group.

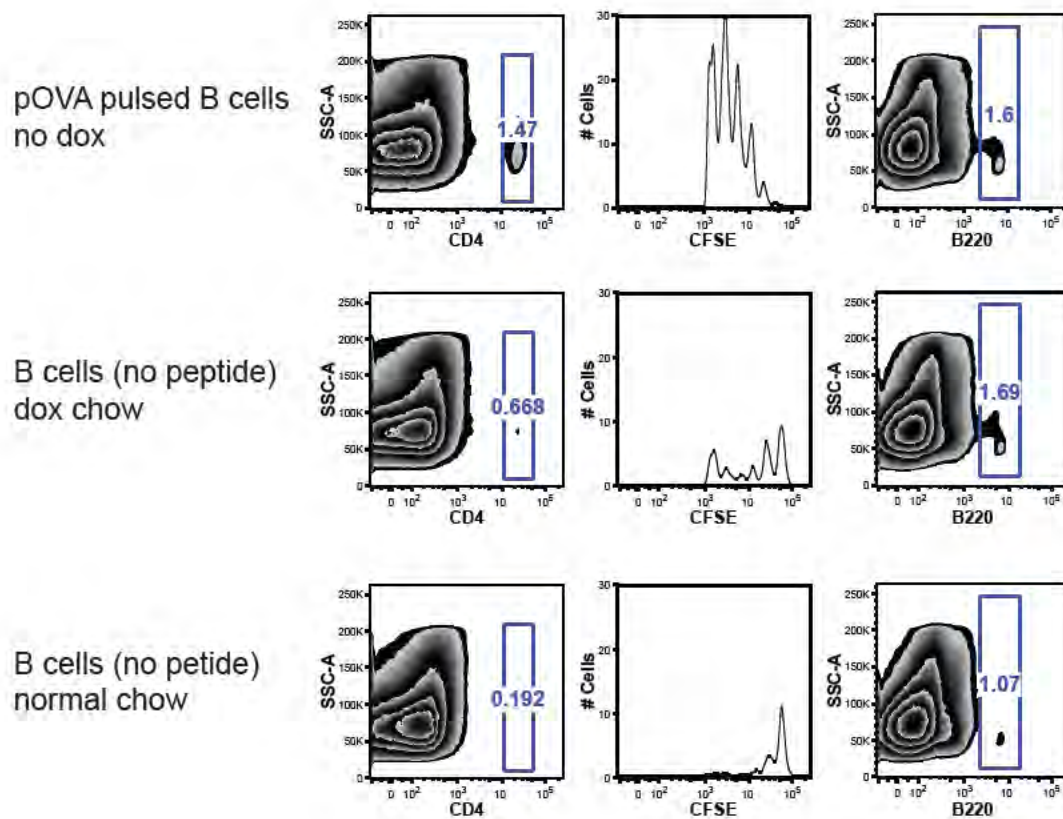


Figure 2.2. B cell expression of TGO can drive DO11 proliferation.

Figure 2.3. Activated Th2, but not naïve autoreactive T cells can elicit autoantibody production in li-TGO mice.

(A) Intracellular cytokine staining from *in vitro*-differentiated DO11 Th2 cells cultured in the presence of irradiated syngeneic APCs with or without ova peptide. Plots show cells within the DO11+ gate. <APC-A> and <PE-A> are used as labels when APC or PE conjugated antibodies were omitted.

(B) 5×10^6 DO11 Th2 cells were adoptively transferred into sublethally irradiated (4 cGy) li-TGO mice fed dox chow. Spleens were harvested at 4 weeks and stained for CD4 and the DO11 TCR (KJ1-26) then analyzed by flow cytometry.

(C) 5×10^6 naïve DO11 or DO11 Th2 cells were adoptively transferred into sublethally irradiated (4 cGy) li-TGO mice fed normal or dox chow. Mice were cheek bled at 6 weeks and serum was analyzed by HEp2 ANA assay. Images are representative of 4 experiments with 3-5 mice per group in each experiment.

(D) Total antibody isotype concentration in li-TGO sera post T cell transfer was quantified by ELISA. Each point represents an individual mouse.

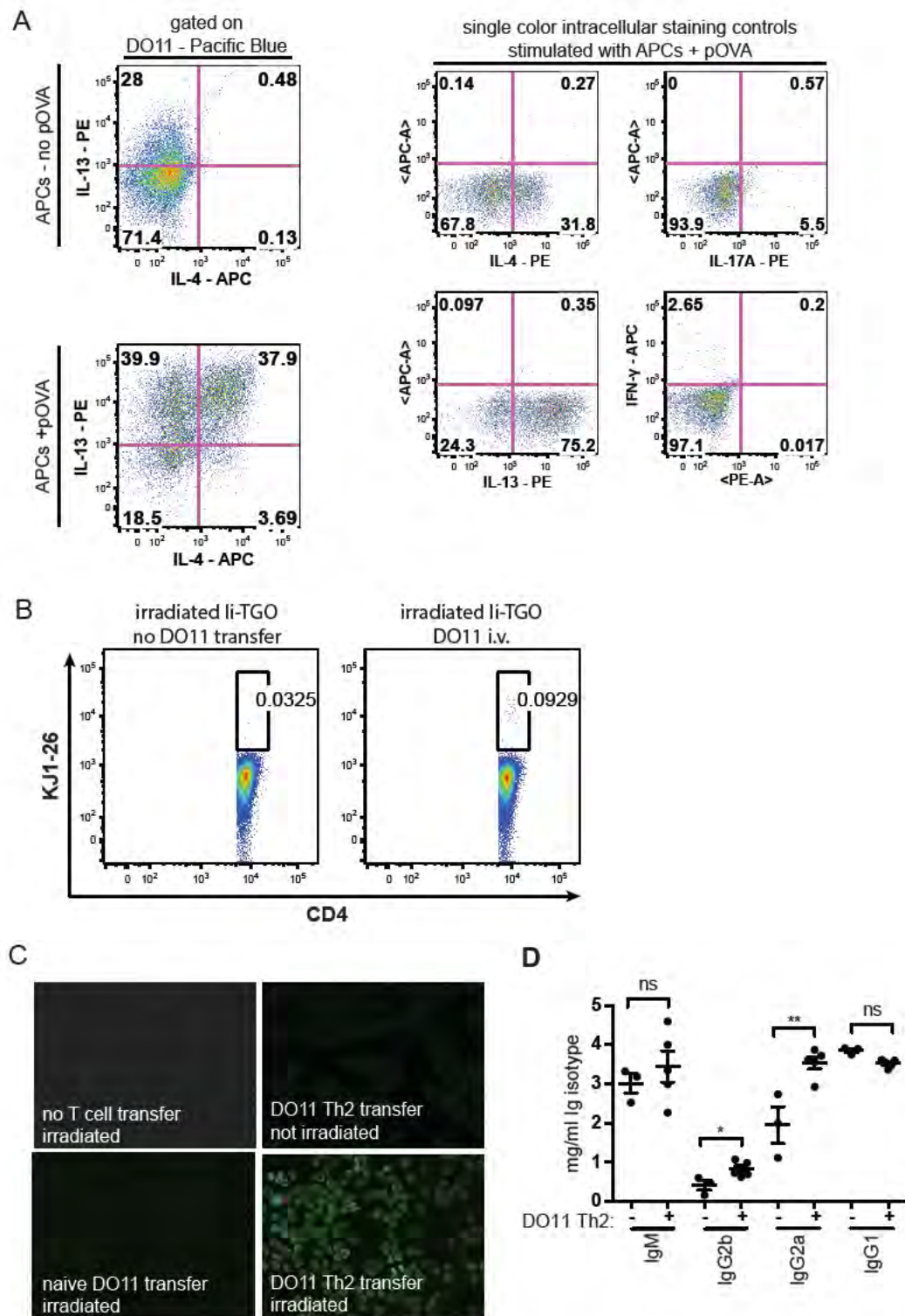


Figure 2.3. Activated Th2, but not naïve autoreactive T cells can elicit autoantibody production in li-TGO mice.

results show that activated T cells can selectively activate autoreactive B cells present in the normal repertoire through cognate interactions.

Non-induced TGO expression by radioresistant APCs drives extensive Treg development.

At least two studies have shown that autoantibody production is dampened in mice immunized with self-Ag and adjuvant for a tissue-specific transgenic Ag if Ag-specific Tregs are present (Gotot et al., 2012; Ludwig-Portugall et al., 2009). Similarly, Tregs can decrease activation of anti-dsDNA B cells in response to T cell help (Seo et al., 2002). Moreover, in previous studies, an unexpectedly high frequency of Treg cells were found in the thymus and skin draining lymph nodes of non-induced TGO mice crossed to DO11 mice, consistent with thymic TGO expression even in the absence of dox (Rosenblum et al., 2011). Therefore we considered the possibility that Tregs could limit autoreactive B cell activation in unirradiated li-TGO mice. I confirmed these results in our lab by generating DO11 x TRE-TGO and DO11 x li-TGO crosses. Surface and intranuclear staining showed a marked increase in KJ1-26⁺ (DO11 anti-clonotype) CD25⁺Foxp3⁺ cells in DO11 x TRE-TGO mice. Frequencies of Tregs in the KJ1-26⁻ compartment were within the normal range (Figure 2.4A).

To determine whether the number of DO11 Tregs would increase in response to increased expression of TGO, I placed DO11 x li-TGO mice on dox chow or maintained them on normal chow for 7 days. The total number of Tregs increased by roughly two-fold, and the total number of DO11 T cells increased by

an average of 50% (Figure 2.4B). DO11 x li-TGO mice treated with dox chow exhibited a four-fold increase in the frequency of DO11 T cells expressing the marker of activation/Ag experience, CD69, and many co-expressed the lymph node-homing molecule CD62L. Neither non-DO11 T cells in the same hosts, nor CD8⁺ T cells increased CD69 expression (Figure 2.4C). These data show that TGO can induce activation of Ag-specific T cells *in vivo*, and that TGO upregulation further expands Ag-specific Tregs.

To determine the relative contribution of radioresistant antigen presenting cells (rAPCs) compared to hematopoietically derived APCs (hAPCs) to Treg induction, we generated BM chimeras using T-depleted bone marrow obtained from either DO11 mice or [DO11 x TRE-TGO] F1 mice to reconstitute lethally irradiated BALB/c or TRE-TGO recipients. In the BALB/c hosts, the majority (>80%) of thymic CD4 single positive (SP) T cells expressed the DO11 TCR, regardless of whether the donor BM was DO11 or DO11 x TRE-TGO, and only a small frequency (<0.5%) of these cells expressed Foxp3. By contrast, in the TGO hosts, a lower percentage of the CD4 SP T cells were KJ1-26⁺, and of these only 15-22% were Foxp3⁺. In the spleen, again both DO11 → BALB/c and DO11 x TRE-TGO → BALB/c chimeras had very few KJ1-26⁺ Tregs and relatively normal proportions of KJ1-26⁻ Tregs. However, in the TGO hosts, the vast majority of DO11 T cells appear to have been deleted since less than 20% of splenic CD4⁺ T cells were KJ1-26⁺. Moreover, of the remaining KJ126⁺ cells, approximately one-third to one-half expressed Foxp3 (Figure 2.5A-B). These

Figure 2.4. Ectopic expression of self-Ag drives extensive Treg development.

(A) Single-cell suspensions from thymi, spleens, and lymph nodes of DO11 and DO11 x TRE-TGO mice were stained for CD4, CD8, CD25 and Foxp3 and analyzed by flow cytometry. The flow cytometry plots shown were first gated on CD4 single positive events, and are representative of four DO11 mice and six DO11 x TRE-TGO mice.

(B) DO11 x li-TGO mice were maintained on normal chow or fed dox chow for 7 days. Spleens were harvested and stained for CD4, DO11 TCR, CD25, and Foxp3. Data are representative of three experiments with 3 mice per group. Bars represent SEM.

(C) Splenocytes from DO11 x li-TGO mice on or off dox for 7 days were stained for CD62L and CD69 after gating on indicated T cell populations. Gates shown above plots. Data are representative of three experiments with 3 mice per group.

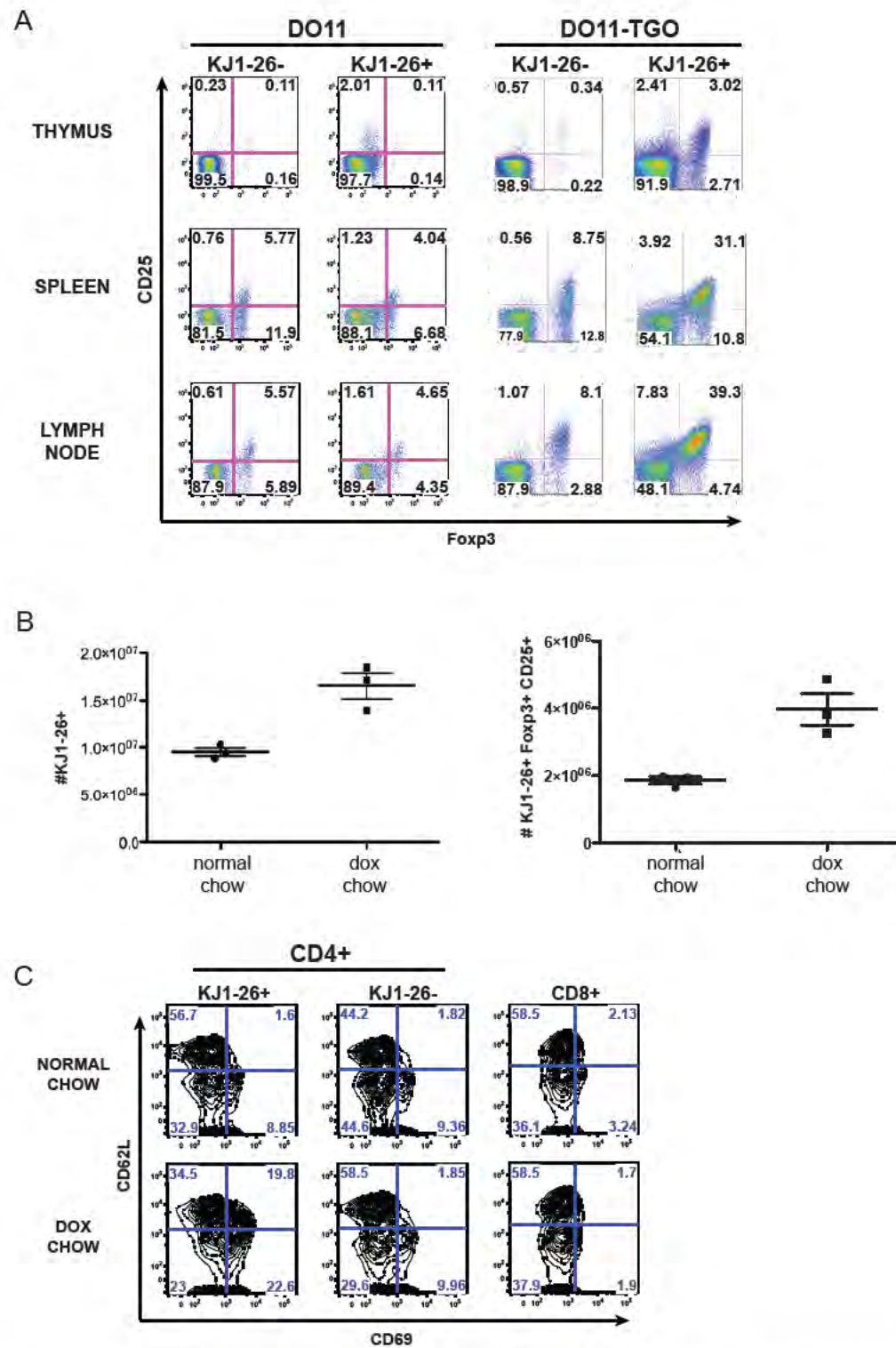


Figure 2.4. Ectopic expression of self-Ag drives extensive Treg development.

Figure 2.5. Non-induced expression of TGO by radioresistant APCs drives extensive DO11 deletion and proportional Treg increase.

(A) Bone marrow chimeras were generated using lethally irradiated BALB/c or TGO hosts reconstituted with DO11 or DO11 x TRE-TGO bone marrow. At 12 weeks, thymi and spleens were analyzed for CD4, CD8, DO11 TCR (KJ1-26 antibody), CD25, and Foxp3. The flow cytometry plots shown were first gated on CD4 SP events. These data are representative of two experiments with 3-4 mice per group.

(B) Tabulation of data shown in (A). The graph was generated from one experiment with 3 mice per group, and is representative of two experiments. Grey bars represent BALB/c hosts and black bars represent TGO hosts. Error bar shows SEM.

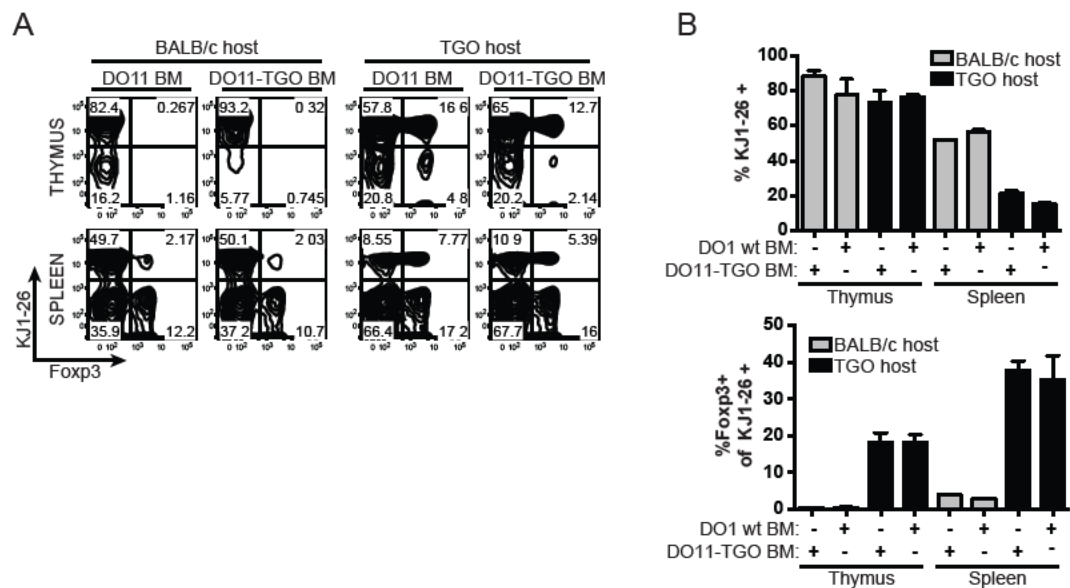


Figure 2.5. Non-induced expression of TGO by radioresistant APCs drives extensive DO11 deletion and proportional Treg increase.

data demonstrate that TGO expression in mice not given dox, and therefore not sufficient to induce the proliferation of mature DO11 T cells, could nevertheless: (a) lead to the deletion of developing DO11 Tg T cells and the proportional expansion of Tg-negative CD4⁺ T cells; and (b) induce the differentiation and/or expansion of DO11 Tg⁺ Tregs. The data further indicated a negligible role for hematopoietically derived APC in this process.

Absence of self-Ag bearing stromal cells is permissive to persistent autoreactive T cell activation and enhanced autoreactive B cell activity.

The induction of lupus-like disease in Th2→li-TGO mice required irradiation, which may have provided functional inactivation of a regulatory population. Taken together with BM chimera data showing that rAPC-derived Ag was largely responsible for Treg generation (Figure 2.5), we next wanted to address whether the magnitude or specificity of B cell ANA production is limited by Ag-specific Tregs that could be driven by stromal-derived TGO. To this end, we generated BM chimeras with negligible numbers of TGO-specific Tregs by reconstituting lethally irradiated non-Tg BALB/c mice with li-TGO bone marrow stem cells [li-TGO→BALB/ chimeras]. We also reconstituted lethally irradiated li-TGO mice with li-TGO stem cells [li-TGO→li-TGO chimeras]. Twelve weeks after reconstitution, chimeric mice were placed on dox to induce TGO expression peripherally, but they were not sublethally irradiated as in the experiment described in Figure 2.3. The chimeric mice were then injected with either naïve

DO11 cells, or DO11 Th2 cells, or left uninjected. Six weeks later, serum samples collected from the mice were assayed for the presence of ANAs. ANAs only developed in mice adoptively transferred with DO11 Th2 cells, suggesting that self-reactive (DO11) naïve T cell activation was effectively controlled even in the absence of self-Ag specific Tregs. However, the li-TGO→BALB/c chimera hosts developed higher ANA titers than the li-TGO→li-TGO chimera hosts (Figure 2.6). Unexpectedly, the frequency of activated non-Tg CD4⁺ T cells was similar in both BALB/c hosts and li-TGO hosts injected with Th2 cells (Figure 2.7A), while B cell CD86 and MHC-II was markedly elevated in BALB/c hosts, but not TGO hosts (Figure 2.7B; CD86 not shown). These data indicate that Ag specific Tregs may tune the quality of autoreactive B cell interactions with autoreactive T cells, even though they apparently fail to suppress T cell activation following an influx of activated self-reactive T cells.

Activation and elaboration of Ag-specific SLO-resident Tregs are preferentially activated by rAPCs.

The foregoing findings suggested a role for stromal self-Ag presentation in the control of aberrant activation and ANA production by B cells. Because B cell activation and ANA production were reduced in the presence of TGO-expressing rAPC, we wanted to determine whether the Treg phenotype was enhanced by peripheral expression of TGO after development and poise Tregs to suppress autoreactive B cells. To probe this question, we generated bone marrow

Figure 2.6. Absence of self-Ag bearing stromal cells is permissive ANA production by autoreactive B cells without sublethal irradiation.

li-TGO→BALB/c and li-TGO→li-TGO bone marrow chimeras were created and reconstituted for 12 weeks. At this time, the mice were not re-irradiated, they received an adoptive transfer of 5×10^6 DO11 Th2 cells or were not injected. At 6 weeks, sera from these mice were analyzed by HEp2 ANA assay. Blinded scoring was performed using a 0-4 scale relative to BALB/c-Fas^{lpr/lpr} serum. Combined data from two experiments is shown. A representative image to reflect the staining pattern is shown to the right of the graph.

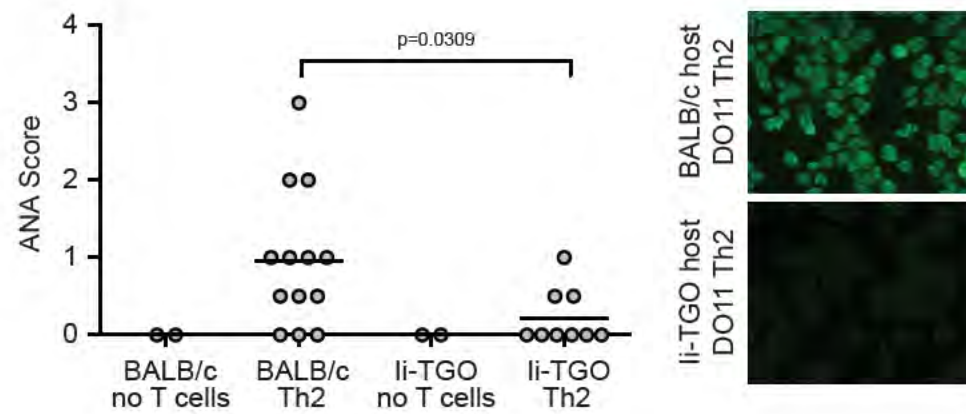


Figure 2.6. Absence of self-Ag bearing stromal cells is permissive to ANA production by autoreactive B cells without sublethal irradiation.

Figure 2.7. Absence of self-Ag bearing stromal cells is permissive to increased B cell activation.

Spleens were harvested from li-TGO→BALB/c and li-TGO→li-TGO bone marrow chimeras that were injected with naïve DO11 T cells or DO11 Th2 cells 12 weeks before. Box plots show 5 mice per group and are representative of two experiments. Flow plots are representative results from each group.

(A) T cells were stained for markers of antigen experience/activation and analyzed by flow cytometry. The KJ1-26⁻ population is shown.

(B) B cells were stained for MHC-II. The gate for MHC-II^{high} cells was set against a normal BALB/c control spleen.

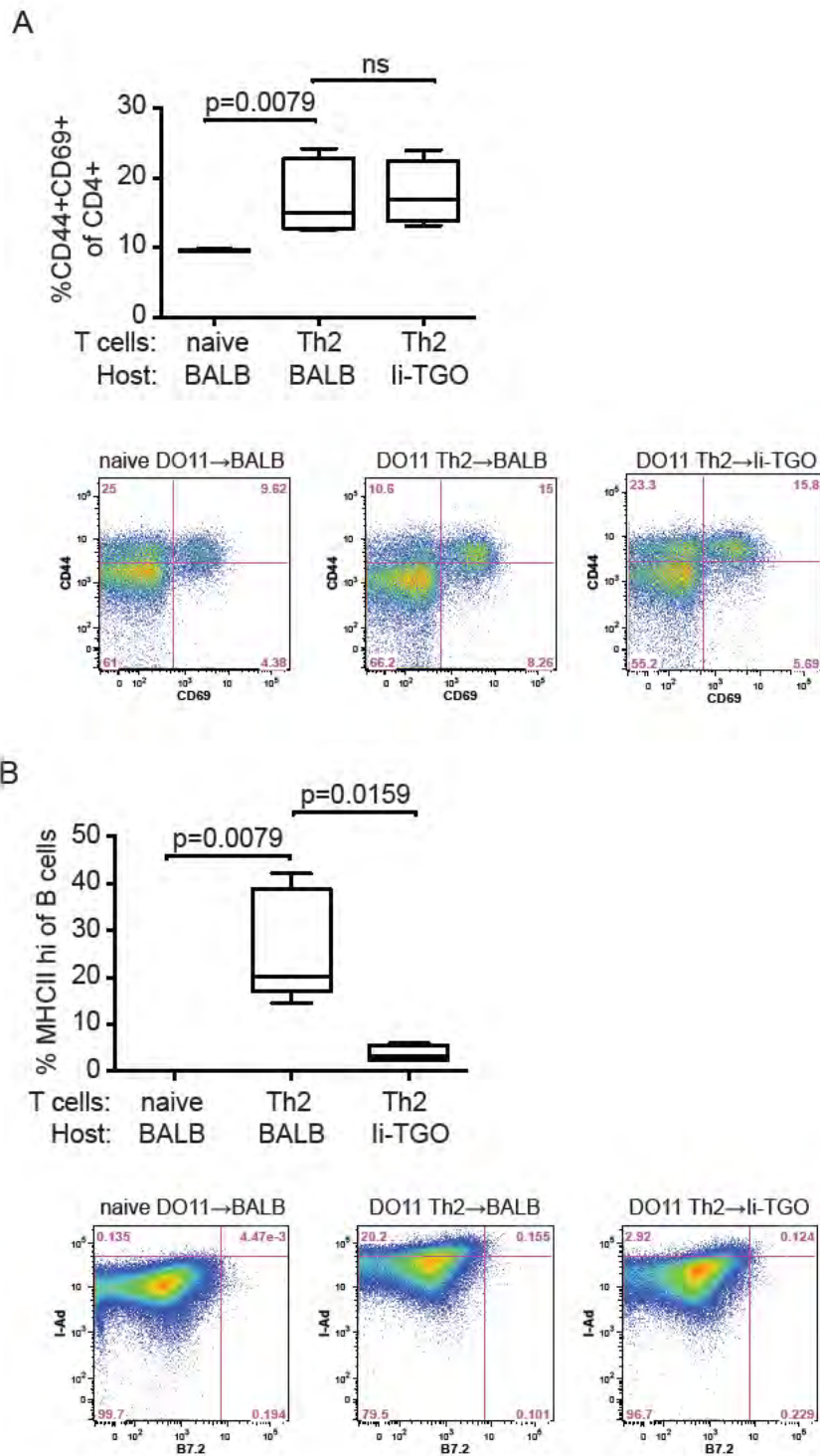


Figure 2.7. Absence of self-Ag bearing stromal cells is permissive to increased B cell activation.

chimeras using DO11 x li-TGO F1 bone marrow stem cells to reconstitute lethally irradiated BALB/c or li-TGO hosts. Twelve weeks after reconstitution, the mice were either kept on normal chow or placed on doxycycline chow for 7 days. In accordance with our previous experiments (Figure 2.5), in both the normal chow and dox chow groups, the frequency and number of KJ1-26⁺ DO11 cells was decreased in li-TGO hosts relative to BALB/c hosts, and a large fraction of DO11 cells in the li-TGO hosts co-expressed the Treg markers CD25 and Foxp3. In the presence of dox, the total number of DO11 cells in both BALB/c and li-TGO hosts increased (Figure 2.8A-B), but remarkably, the number of DO11 Tregs only increased in li-TGO hosts (Figure 2.8C). Moreover, in the presence of dox, the percentage of DO11 cells expressing both the activation marker CD69 and the lymph node homing receptor CD62L increased in both BALB/c hosts and li-TGO hosts. Quite interestingly, over half of the CD69⁺CD62L⁺ T cells were also CD25⁺Foxp3⁺ Tregs in li-TGO hosts, but less than 10% of CD69⁺CD62L⁺ T cells in BALB/c hosts were Tregs (Figure 2.8D-E). Both CD69 and CD62L expression by Tregs has been associated with more effective anti-inflammatory function in other models of sterile inflammation (Ermann et al., 2005; Fu et al., 2004; Szanya et al., 2002). Our data indicate that a radioresistant peripheral host cell population bearing self-Ag most effectively activates and expands Tregs which home preferentially to SLOs by expression of the SLO homing molecule CD62L and could potentially suppress autoreactive B cell activation and ANA production.

Figure 2.8. Self-Ag presentation by radioresistant APCs drives activation and elaboration of SLO-tethered Tregs.

DO11 x li-TGO bone marrow was used to reconstitute lethally irradiated BALB/c and TGO hosts. At 12 weeks, the mice were either maintained on normal chow or placed on dox chow. The mice were sacrificed 7 days later and spleen cell suspensions were stained and analyzed by flow cytometry. Data are the combined results of two experiments. Bars represent SEM.

- (A) Plot of the total number of cells staining positively with KJ1-26 within the CD4⁺ gate.
- (B) Plot of the frequency of cells staining positively with KJ1-26 within the CD4⁺ gate.
- (C) Plot of the total number and frequency of CD25⁺Foxp3⁺ cells within the KJ1-26⁺ gate.
- (D) Plot of the total number and frequency of CD25⁺Foxp3⁺ cells within the KJ1-26⁺ CD62L⁺ CD69⁺ gate.
- (E) Representative flow cytometry plots from DO11 x li-TGO→BALB/c or DO11 x li-TGO→TGO chimeras on dox.

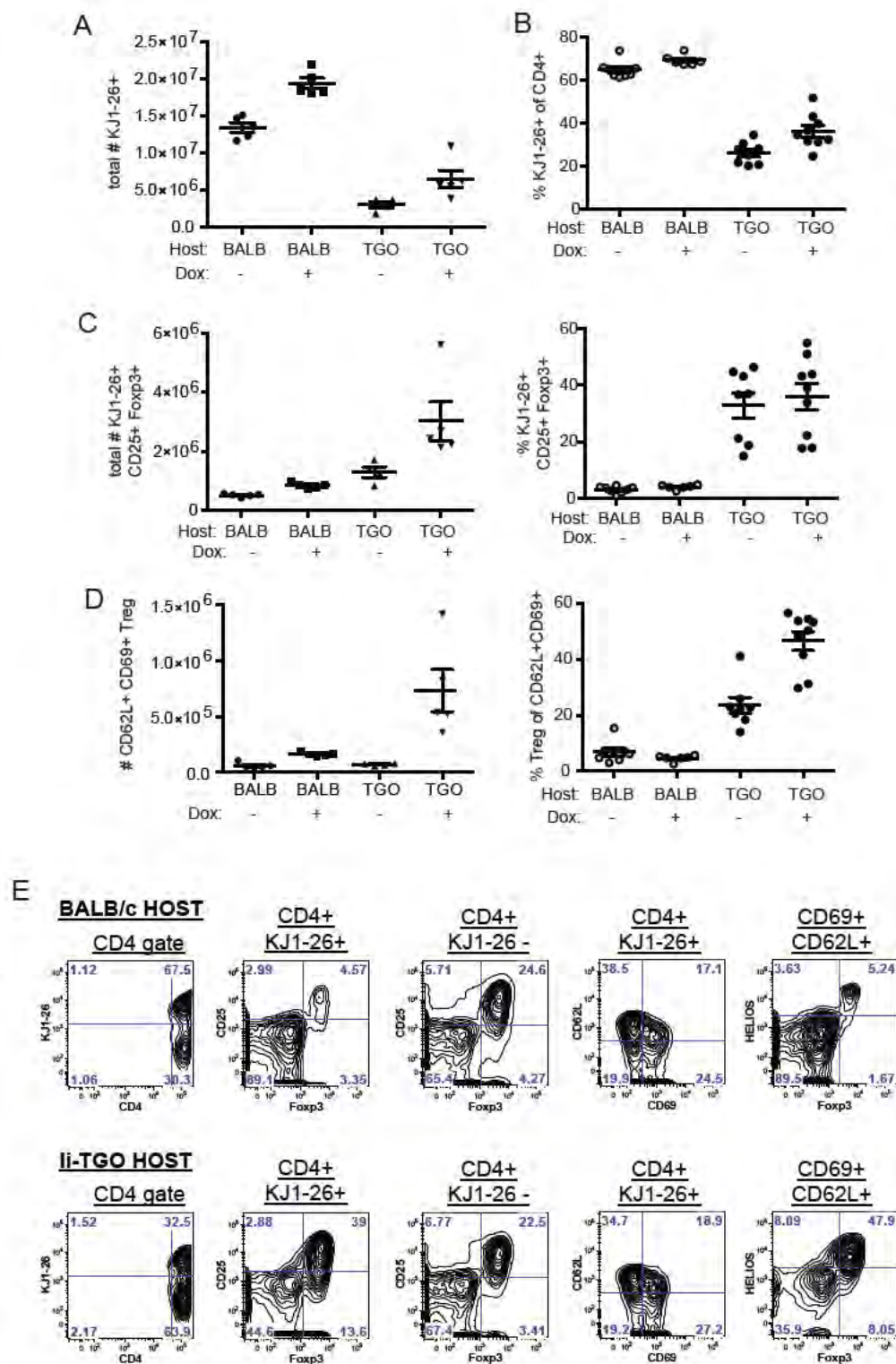


Figure 2.8. Self-Ag presentation by radioresistant APCs drives activation and elaboration of SLO-tethered Tregs.

In order to determine whether radioresistant lymph node stroma could (1) expand Tregs and (2) sustain or enhance CD62L expression on activated Tregs, we co-cultured CD25-purified cells from the spleen of a naïve DO11 x li-TGO mouse with CD45⁻ (LNSC) or CD45⁺ cells from an li-TGO mouse. Purity of the CD45⁻ population was routinely >95% after magnetic depletion of CD45⁺ cells. Unfortunately, the addition of dox to LNSC cultures caused cell death—even at concentrations lower than what our lab has previously used to induce TGO in murine embryonic fibroblasts. For this reason, the data I am including is from cultures in which pOVA was added to the CD45⁻ and CD45⁺ cells to stimulate DO11 cells. After 5 days of culture, I stained non-adherent cells from the cultures for CD25 and Foxp3. Interestingly, in the absence of pOVA, the majority of DO11 x li-TGO T cells did not express Foxp3 (Figure 2.9A), although >80% expressed Foxp3 prior to being cultured. In the presence of pOVA, regardless of whether CD45⁻ or CD45⁺ cells were used as APCs, the frequency of Tregs was comparable to the pre-culture Treg frequency (Figure 9B). This suggests that many of the Tregs in DO11 x li-TGO mice do not stably express Foxp3 without Ag-stimulation. Despite the potential mixed stability of the Tregs in culture, LNSC cause upregulation of CD62L on Tregs only in the presence of pOVA, and CD45⁺ APCs promoted downregulation of CD62L (Figure 2.9B-C).

To determine whether LNSC-mediated CD62L upregulation on Tregs was a Treg-specific phenomenon, we co-cultured CD45⁻ stromal cells (purity shown in 2.9D) or CD11c⁺ cells (largely dendritic cells (DC)), obtained from pooled lymph

Figure 2.9. Cognate Ag presentation by LNSC reinforces Treg capacity for lymph node homing.

(A) CD25-purified DO11 x li-TGO T cells were cultured with CD45⁻ and CD45⁺ cells from lymph nodes with or without pOVA. At day 5, cultures were stained for Treg markers and analyzed by flow cytometry.

(B) Cells from (A) were also stained for CD62L, and the frequency of CD62L^{high} cells is plotted. Data in (A) and (B) are the combined results of two independent experiments. Bars show SEM.

(C) Histogram depicting log₁₀ fluorescence of CD62L on DO11 Tregs after culture described in (A).

(D) Representative plot of CD45⁻ cell purity after CD45 depletion.

(E) CD45⁻ stromal cells or CD11c⁺ cells purified from pooled lymph nodes of li-TGO mice were cultured with CD4⁺ T cells from DO11-Foxp3^{eGFP} x li-TGO mice. Plot shows the frequency of CD62L^{high} cells within the KJ1-26⁺ eGFP⁺ gate.

(F) Plot shows the frequency of CD62L^{high} cells within the KJ1-26⁺ eGFP⁻ gate from the same cultures as (E). The results shown are pooled from two experiments.

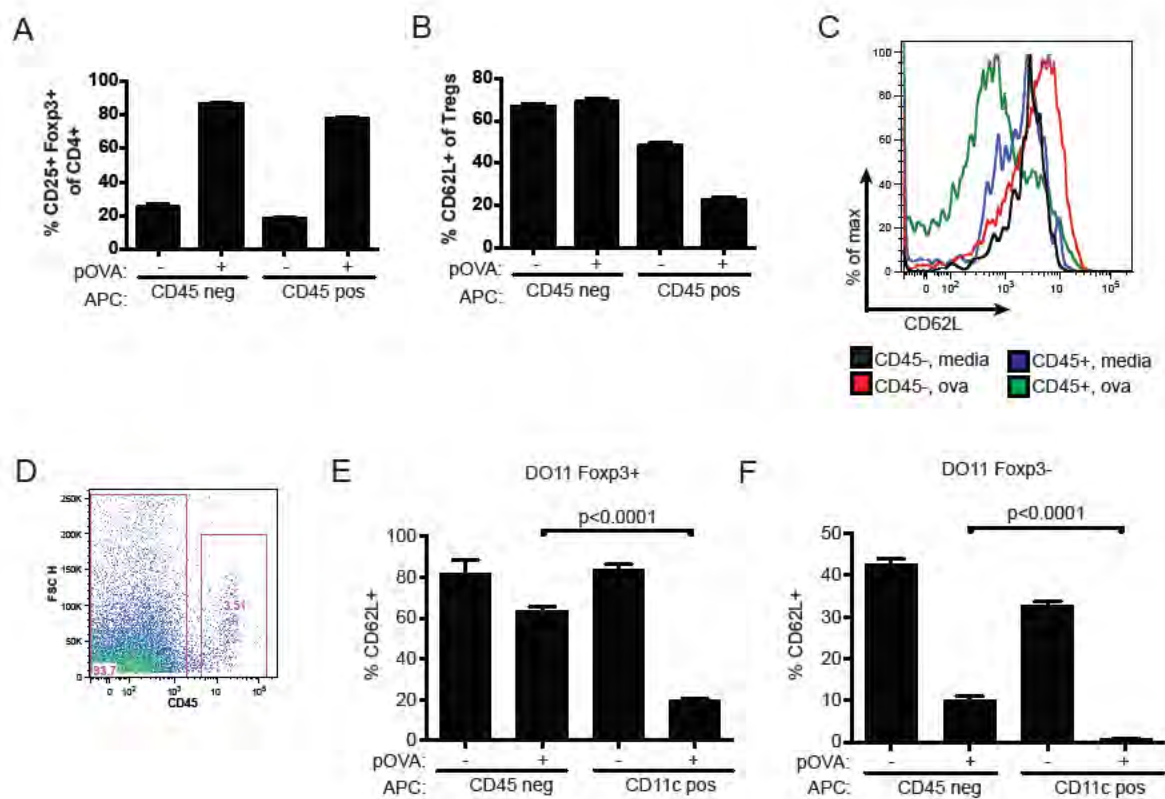


Figure 2.9. Cognate Ag presentation by LNSC reinforces Treg capacity for lymph node homing.

nodes of li-TGO mice, with CD4⁺ T cells from DO11-Foxp3^{eGFP} x li-TGO mice. Activation by LNSC led to a modest decrease in CD62L by eGFP⁺ Tregs in the presence of pOVA, while CD11c⁺/T cell co-cultures led to a striking decrease in the frequency of CD62L⁺ Tregs (Figure 2.9E). Strikingly, although stroma-directed reinforcement of CD62L expression was robust in the Treg population, a significantly lower frequency of eGFP⁻ (non-Treg) T cells expressed CD62L (Figure 2.9F). Thus we have identified a previously unrecognized player in dominant tolerance, the SLO stromal cell, which both activates and poises Foxp3⁺ Tregs to suppress activation of unprimed autoreactive T and B cells in spleen or lymph node through sustained expression of CD62L following activation.

Discussion

Chronic positive feedback loops due to sustained T cell-B cell interaction may drive systemic autoimmunity in diseases such as lupus. In animal models of lupus, T cells promote IgG production from spontaneously activated autoreactive B cells, and B cells promote activation of autoreactive T cells. Our work presented here broadens the understanding of how aberrantly activated autoreactive T cells may facilitate breakage of B cell tolerance. Notably, our experiments examine the behavior of normal B cell repertoire, presumably including a small frequency of autoreactive B cells, which are availed of cognate interactions with T cells. Autoantibody production by autoreactive B cells and

stimulation via CD40L and IL-21—mechanisms by which T cells enhance antibody responses—can be increased even by the provision of non-Ag-specific T cell help (Sweet et al., 2011). We show that even with Ag-specific interactions, naïve T cells could not drive ANA production from endogenous autoreactive B cells. DO11 T cells from naïve mice divide in li-TGO mice, but did not stimulate ANA production under conditions where activated DO11 T cells could stimulate ANAs. Previous work has shown that naïve B cell Ag presentation to a naïve T cell results in hyporesponsiveness of the T cell (Fuchs and Matzinger, 1992), and it is likely that naïve DO11 T cells became anergic or proliferated abortively (Townsend and Goodnow, 1998). This seems likely, since only a small number of DO11 T cell were recovered from spleens on unirradiated li-TGO mice. From the available data, we cannot be certain that naïve T cells engage li-TGO B cells in vivo, since debris from li-TGO B cells could have been presented to DO11 T cells by a Rag2^{-/-} host APC. Although cognate interactions may not be required for Tg autoreactive B cells to receive T cell help, the nature of the autoAgs used to activate Tg B cells could have an impact on the B cell's ability to respond.

Autoreactive T cells specific for autoAgs that are commonly recognized by B cells are present at low levels in the T cell repertoire (Dumortier et al., 2000; Yan and Mamula, 2002), and T cells from autoimmune mice, which are presumably primed, stimulate autoantibody production from anti-snRNP Tg B cells (Yan and Mamula, 2002). Activated DO11 Th2 cells did elicit ANA production in sublethally irradiated li-TGO mice, and this was likely due to

production of cytokines such as IL-21 and IL-4 (data not shown) and increased CD40L relative to naïve cells. However, sublethal irradiation was required for li-TGO mice to become ANA+ even after transfer of activated DO11 Th2 cells. There are several potential explanations for this finding. First, irradiation provided immunological “space” for the large influx of activated cells, and the contraction of this population was therefore less sharp. A second possibility is that irradiation provided apoptotic debris due to widespread lymphocyte death that served as autoAg. This possibility is less likely, since we have no reason to believe that this cell debris persisted for the number of days required for B cell re-emergence in the periphery (~11-13 days for follicular B cells). A third possibility is that sublethal irradiation inactivated a regulatory cell population that suppressed DO11 Th2, autoAg-specific B cells, or both.

We determined that expression of TGO by radioresistant host cells could drive Treg differentiation. This effect was in part a proportional increase due to negative selection of DO11 T cells. However, the administration of dox to DO11 x li-TGO mice led to an increase in DO11 Tregs that could have been the result of peripheral induction of Tregs or modest proliferation of thymically derived Tregs. The former seems more likely, since the majority of Tregs from DO11 x li-TGO mice lost Foxp3 expression, suggesting that these cells were a less stable, peripherally induced population. However, these data suggest that increasing TGO expression could induce Ag-specific Tregs or drive proliferation of existing Tregs. We first approached this question by creating chimeric mice that have

virtually no TGO-specific Tregs, but could present TGO on hAPCs (li-TGO→BALB/c), as well as chimeric mice that most likely had a population of TGO-specific Tregs, where TGO can be expressed by both rAPCs and hAPCs. Without sublethal irradiation, li-TGO→BALB/c chimeras became ANA+ after DO11 Th2, but not naïve DO11 injection, consistent our hypothesis that TGO presentation by rAPCs induced a radiosensitive regulatory cell population.

li-TGO chimera experiments revealed that T and B cell activation was not suppressed evenhandedly in TGO hosts. We found that expression of the marker of Ag experience CD44 and activation marker CD69 were upregulated on CD4⁺ KJ1-26⁻ T cells in both li-TGO→BALB/c and li-TGO→li-TGO chimeras that were given dox and injected with Th2 cells, but this was not observed after injection of naïve cells (Figure 7). Whether these activated T cells are oligoclonal or autoreactive is not known at this time, however, it suggests that autoimmunity in li-TGO→li-TGO chimeras was not prevented at the level of T cell activation. Interestingly, MHC-II upregulation correlated with increased ANA titer in the li-TGO→BALB/c cohorts that were injected with Th2s. Decreased MHCII on B cells suggests dominant tolerance, potentially exerted by Tregs, suppressed B cell activation. Ideally, we would deplete Tregs specifically to test this hypothesis, and to this end, we plan to cross li-TGO onto a Foxp3-DTR mouse line. Additionally, experiments to determine whether Tregs are eliminated or exhibit functional impairment in sublethally irradiated li-TGO mice should be performed.

TGO expression by rAPCs led to activation of DO11 Tregs (using CD69 expression as criteria for activation) while maintaining expression of CD62L, which is usually downregulated after priming of naïve T cells (although it can later be upregulated) (Figure 8). CD62L⁺ Tregs are potent suppressors of graft-versus-host disease and in a model of type 1 diabetes, most likely because they home preferentially to lymph nodes and can suppress primary immune responses (Ermann et al., 2005; Fu et al., 2004; Szanya et al., 2002). Since it was possible that a radioresistant, hematopoietically derived APC was responsible for the CD62L⁺ CD69⁺ DO11 Treg phenotype in DO11 x li-TGO BM chimeras, we used an *in vitro* culture system to examine stromal cell/Treg interactions. Upregulation of CD62L on Tregs in these cultures suggests that the SLO stromal cells can activate and poise Foxp3⁺ Tregs to suppress activation of naïve autoreactive T and B cells in spleen or lymph node through sustained expression of CD62L following activation. This phenomenon appears to be Treg specific, and the large presence of non-Tregs in CD4-purified cell/stromal cell cultures slightly abrogated CD69 maintenance on Tregs (Figure 9). This may have been due to production of effector cytokines such as IFN- γ , which has been shown to act on LNSCs and enhance production of iNOS to regulate T cell division (Fletcher et al., 2010; Fletcher et al., 2011; Khan et al., 2011; Lukacs-Kornek et al., 2011; Siegert et al., 2011). I considered the possibility that sub-lethal irradiation decreased the functional capacity of LNSC to regulate the proliferation of adoptively transferred DO11 Th2 cells. If this were the case, it

could be a partial explanation for why irradiation was required to elicit ANA production in Th2→li-TGO mice (Figure 3). This notion seems less likely given the results from DO11 x li-TGO BM chimeras, where rAPC were capable of expanding and activating DO11 Treg even after lethal irradiation. The relative ability of LNSC to regulate specific Th subsets remains to be seen, but our data suggest that Treg:LNSC interactions and non-Treg:LNSC may have markedly different functional consequences.

The results shown in this chapter demonstrate that T cell activation is an important checkpoint in the prevention of autoantibody production, that autoreactive T cell activation is sufficient for driving autoantibody production by autoreactive B cells, and that Ag-specific tolerance directed by rAPC—most likely Tregs—most effectively control autoantibody responses.

Materials And Methods

Mice

BALB/c mice were purchased from the Jackson Laboratory and rested in our colony for at least one week before use. Foxp3^{eGFP} and DO11.10 mice were bred in-house after purchase from Jackson Laboratory. DO11.10 *Rag2*^{-/-} mice were bred in-house after purchase from Taconic. All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School. In all experiments, the mice were age-matched

within two weeks difference in date of birth.

Doxycycline administration

Mice were fed ad lib with doxycycline chow (200mg dox per kg of chow; Bioserv).

Irradiation and bone marrow chimeras

Mice were irradiated using a Cesium-137 irradiator in rotating containers. For adoptive transfers of mature T cells, mice were dosed with 400 rads and injected the following day. For lethal irradiation prior to bone marrow transfer, mice were dosed with 800 rads and injected the following day. Bone marrow was flushed from femurs and tibias of naïve donors using a 25 gauge needle and syringe and cold sterile PBS + 2% FBS. The cells were spun-down and re-suspended in complete RPMI. T cells were depleted using supernatants from hybridomas secreting GK1.5 (anti-CD4) and (53-67.2) anti-CD8 and complement. The cells were washed twice in complete RPMI and twice in sterile PBS. 10×10^6 bone marrow cells were injected into mice that had received 800 rads on the previous day.

Flow cytometry

Single cell suspensions were surface or intracellular stained with combinations the following antibodies: I-A/I-E-FITC, CD3 (clone 145-2C11), CD4 (clone RM4-5), CD8 α (clone 53-6.7), CD25-PerCP-Cy5.5 (clone PC61.5), CD69-efluor450, CD62L-APC-efluor780, B220, Foxp3-PE (clone FJK-16s) all from eBioscience,

and CD45-APC (Miltenyi). For Foxp3 staining, cells were fixed and permeabilized using the Foxp3 Transcription Factor staining buffer kit (eBioscience) according to the manufacturers instructions.

***In vitro* T cell differentiation and transfer**

CD4⁺ T cells were purified from DO11 lymph node and spleen cell suspensions by positive selection (BD iMag CD4 beads; manufacturer's instructions). RPMI medium 1640 (cRPMI; Life Technologies, Grand Island, NY), containing 10% FCS (Sigma), 10 μ M 2-mercaptoethanol, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 0.3 mg/ml glutamine (GIBCO) was used for cell culture.

Cells cultures were stimulated with irradiated BALB/c spleen (3,000 rads; 1:5 ratio of T cells to irradiated spleen) for 48-72 hours in the presence of pOVA (1 μ g/ml; Genscript), X63.II4 supernatant (1:4) and, X63.II2 supernatant (2 drops per 5ml). Cells were split on day 2 and then as needed. Cultures were supplements with X63.II2 supernatant each time cells were split. On day 7-9, when T cells had rounded in shape, cultures were re-stimulated with irradiated BALB/c spleen (3,000 rads) for 48-72 hours in the presence of pOVA. 5x10⁶ cells were injected per mouse i.v. "Naïve" DO11 cells were CD4 purified from lymph node and spleen suspensions, washed in PBS, and 5x10⁶ cells were injected per mouse i.v.

CFSE Labeling

Before labeling, cells were washed twice in sterile PBS and re-suspended at a concentration of 5×10^6 /ml. A 5mM stock solution of CFSE (Invitrogen) was diluted in PBS (1:2,000) in a volume equal to the cell suspension. The two populations were combined using a serological pipet and mixed thoroughly. The tube of cells was covered in foil and incubated at 37°C for 5 minutes. The cells were washed three times in PBS + 2% FBS and prepared for adoptive transfer.

HEp2 ANA assay

Sera were diluted 1:50 in PBS and placed on HEp2 ANA slides. BALB/c-Fas^{lpr/lpr} serum and normal BALB/c serum were used as positive and negative controls. After 60 minutes at room temperature, the slides were washed twice in a Coplin jar filled with fresh PBS, with rocking, for 5 minutes. Slides were then stained with anti-mouse IgG-FITC (Southern Biotech). After 30 minutes at room temperature, the slides were washed as above, dipped in tap water, and mounted with ProLong Gold (Invitrogen).

Stromal cell and CD11c⁺ cell isolation

Lymph nodes and spleens were placed in a Petri dish containing pre-warmed RPMI with 2% FBS containing 0.5mg/ml dispase and 0.5 mg/ml collagenase. The tissues were gently mechanically disrupted with frosted glass slides briefly, and then incubated for 15 minutes at 37°C. A sterile P1000 pipet tip was cut with an autoclaved razor to increase the size of the opening. This large-bore tip was

used to disrupt the tissues by pipetting. The enzyme cocktail was removed by pipetting and washed four times with cRPMI. Fresh enzyme cocktail was added to the remaining solid tissue, and this process was repeated twice more.

After four washes, single cell suspensions of lymph nodes and spleen were used for CD11c positive selection or CD45 depletion (Miltenyi; according to manufacturer's instructions).

Statistical methods

Data analysis was performed using Prism (GraphPad) software. p values were calculated using unpaired two-tailed Student's t test. In all figures, ns (not significant) denotes a $p > 0.05$, *, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$.

CHAPTER III

IL-21 Drives Pulmonary Fibrosis through the Induction of Pro-fibrotic CD8⁺ T Cells

Introduction

Fibrosis, defined as the excess production and accumulation of extracellular matrix components, is the final common pathway of many chronic inflammatory conditions (Wick et al., 2013; Wynn, 2004). Despite the prevalence of fibrosis as both a primary disease and a consequence of common chronic diseases such as asthma, sarcoidosis, and heart failure, there are currently no FDA-approved treatments that specifically target fibrogenesis (Wynn and Ramalingam, 2012). Idiopathic pulmonary fibrosis (IPF) is a progressive condition with a poor prognosis and minimal therapeutic options. Fibrotic interstitial lung disease also occurs in autoimmune diseases such as systemic sclerosis (SSc), and rheumatoid arthritis (Wick et al., 2013; Wynn, 2004, 2011; Wynn and Ramalingam, 2012). In these diseases, cells that are beneficial during normal tissue repair, such as macrophages, T cells, and fibroblasts, drive an excessive tissue repair response (fibrosis), leading to organ dysfunction (Wynn, 2004, 2011).

Activation of fibroblasts by TGF- β and consequent collagen production is crucial for both wound healing and fibrosis. Additionally, Th2 cell derived IL-4 and IL-13 are integral components of the wound healing response due to the ability of these cytokines to activate fibroblasts (Wynn, 2004, 2011). IL-13 is also

a potent stimulus for macrophage production of TGF- β (Lee et al., 2001), and can drive lung fibrosis in the absence of other lung injury (Lee et al., 2001). Importantly, Th2-associated cytokines become aberrantly upregulated in fibrosis. Because CD4⁺ T cells have effector functions in murine models of SSc (Radojic et al., 2010; Wallace et al., 1994) and pulmonary fibrosis (Pesce et al., 2006; Wilson et al., 2010), many studies have examined the role of T cell cytokines such as IFN- γ , IL-4 and IL-13 in tissue pathology. Importantly, IL-13 has been linked to human disease and CD8⁺ T cells by a number of studies. IL-13 expression is significantly elevated in IPF (Murray et al., 2013), IL-13 is present in IPF patient lung lavage fluid, fibroblasts from IPF patients are hyper-responsive to IL-13 (Wynn, 2011), and SSc patients were shown to have a predominantly Th2/Tc2-biased T cell pool (CD4⁺ and CD8⁺) (Wynn, 2004). Furthermore, CD8⁺ T cells from SSc patients have been reported to secrete IL-13 and stimulate fibroblasts in an IL-13 dependent manner (Fuschiotti et al., 2013). Lung function decline has also been associated with type 2 skewed CD8⁺ T cells in SSc (Atamas et al., 1999). Nevertheless, neither the actual roles of activated Tc2 cells relative to Th2 cells nor the genesis of Tc2 cells in vivo is well understood.

Development of murine pulmonary fibrosis following bleomycin treatment, a sterile lung injury model, requires both an early Th17 (Oh et al., 2011; Wilson et al., 2010) response, and an IL-13 response (Belperio et al., 2002; Fichtner-Feigl et al., 2006; Jakubzick et al., 2003) which is somewhat more delayed (Wilson et

al., 2010). Asthma, which is often accompanied by subepithelial fibrosis, is a largely Th2-driven disease, and a role for Tc2 cells remains controversial (Das et al., 2006; Miyahara et al., 2004). Fibrosis associated with schistosomiasis also requires IL-13 production, and CD4⁺ T cells are the main producers of IL-13 in these systems (Wynn, 2004). It has therefore been presumed that CD4⁺ T cells are the sole source of IL-13 in sterile fibrosis, although, this has not been definitively shown. However, in a rarely cited report, depletion of CD8⁺ T cells appears to almost completely prevent bleomycin-induced pulmonary fibrosis (Piguet et al., 1989), as does perforin deficiency (Miyazaki et al., 2004). Thus we reasoned that the relative contribution of Th2 and Tc2 cells to the fibrotic process warranted further investigation.

CD4⁺ T cells, including Th17 cells, and NKT cells secrete IL-21. IL-21 is a highly pleiotropic cytokine, which, in combination with other cytokines, amplifies the function of both CD4⁺, and CD8⁺ T cells (Liu and King, 2013; Spolski and Leonard, 2008). Interestingly, IL-21 has been implicated in induction of both Th2 (Frohlich et al., 2007; Pesce et al., 2006) and Th17 (Korn et al., 2007) cells, and as such, could provide a bridge for the progression from a Th17 to a Th2 response in the fibrosing lung. Furthermore, in human fibrotic lung samples, expression of IL-21 receptor (IL-21R) has been detected in lymphocytic infiltrates, suggesting that IL-21 responsive lymphocytes may be involved in lung fibrosis (Parrish-Novak et al., 2000; Parrish-Novak et al., 2002). In mice, IL-21R deficiency attenuated collagen deposition in the liver caused by parasitic infection

(Pesce et al., 2006). Taken together, these data implicate IL-21 as potentially deleterious in pulmonary fibrosis. We now show that IL-21 plays a critical role in the activation and expansion of IL-13 producing Tc2 cells, which in turn function as a critical link between inflammation and fibrosis.

Results

IL-21R signaling drives fibrosis but is dispensable for inflammation after sterile lung injury.

To determine whether IL-21 plays a role in a sterile lung injury model of fibrosis, we compared the effects of intratracheal (i.t.) instillation of bleomycin in wild type C57BL/6 (B6) mice and IL-21 receptor knockout (*Il21r^{-/-}*) B6 mice at days 14 and 21 post treatment. By histological analysis, lung inflammation was comparable between B6 and *Il21r^{-/-}* mice as hematoxylin and eosin (H&E) staining indicated a similar extent of mononuclear cell infiltration, with the majority of the infiltrate being granulocytes (Figure 3.1A). To further compare the B6 and *Il21r^{-/-}* mice, we determined the actual number of cells in bronchoalveolar lavage fluid (BALF) and lung draining (mediastinal) lymph nodes (LDLN) at day 14. In accordance with the histopathology, *Il21r^{-/-}* mice treated with bleomycin had BALF and LDLN cell numbers that were similar to B6 mice treated with bleomycin, but markedly increased compared to PBS-treated control mice (Figure 3.1B). However, there was a striking attenuation in peribronchiolar fibrosis in *Il21r^{-/-}* mice at both days 14 and 21 after bleomycin injury, as detected

Figure 3.1. IL-21R deficiency does not protect from bleomycin-induced lung inflammation.

B6 and *Il21r*^{-/-} mice were treated with bleomycin (bleo; 0.05U/mouse) or PBS (control) i.t. and sacrificed at day 14.

(A) Formalin-fixed, paraffin-embedded lung sections were stained by H&E. Magnification = 20X.

(B) LDLN and BALF cells were collected at day 14 and cell numbers/mouse were determined. Data are compiled from three independent experiments (PBS→B6, n=6; PBS→*Il21r*^{-/-}, n=4; Bleo→B6, n=10; Bleo→*Il21r*^{-/-}, n=11. Bar indicates median ± SEM.

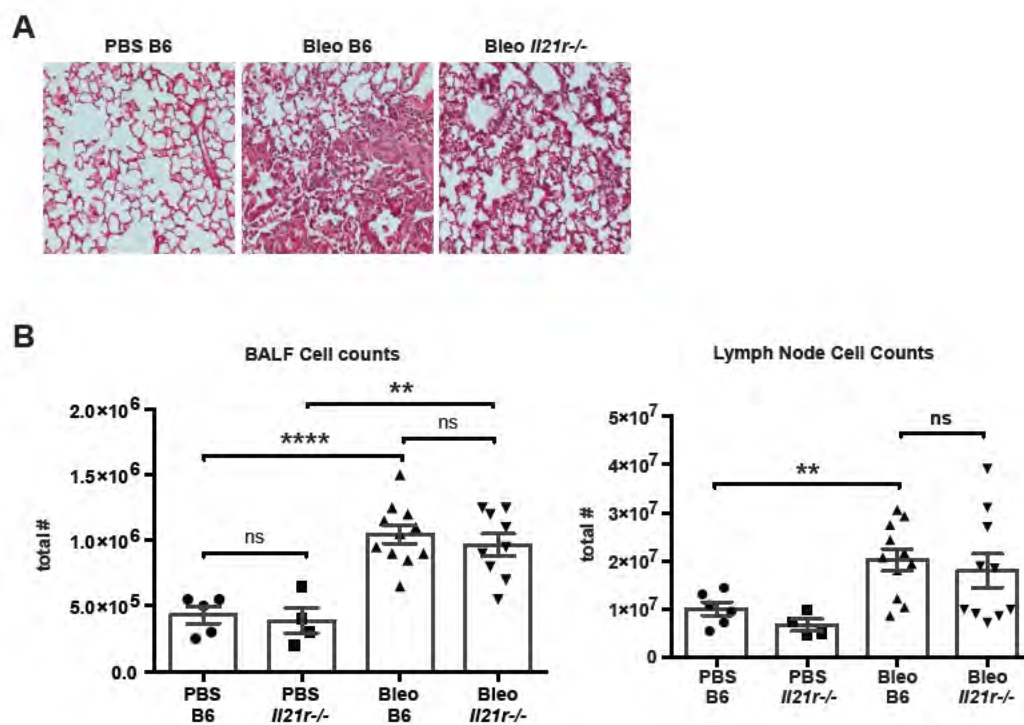


Figure 3.1. IL-21R deficiency does not protect from bleomycin-induced lung inflammation.

by Masson's trichrome staining (Figure 3.2A and data not shown). In addition, quantification of collagen concentration in lung extracts at 14 days by a colorimetric collagen binding assay confirmed that both *Il21r*^{-/-} and IL-21-deficient (*Il21*^{-/-}) mice had significantly decreased collagen deposition in the lung compared to bleomycin-treated B6 mice (Figure 3.2B).

To determine whether fibroblasts in *Il21r*^{-/-} mice became activated after bleomycin injury, lung sections were stained for α -smooth muscle actin (α -smAc), a protein expressed by collagen-producing myofibroblasts upon activation. Remarkably, α -smooth muscle actin was readily detected in the bleomycin injected B6 mice but completely absent from the *Il21r*^{-/-} mice (Figure 3.2C). In addition, survival of *Il21r*^{-/-} mice in response to high-dose bleomycin was prolonged compared to B6 controls (Figure 3.2D). Together, these results identify IL-21 signaling as a critical event in the transition between inflammation and fibrosis.

CD8⁺ T cell recruitment is impaired in *Il21r*^{-/-} mice after sterile lung injury.

Several lines of evidence demonstrate that T cells have an integral role in the transition from inflammation to fibrosis, thus we wanted to determine whether decreased T cell recruitment or activation was responsible for attenuated pulmonary fibrosis in *Il21r*^{-/-} mice. At day 14 post-bleomycin injury, we stained single-cell suspensions of LDLN cells for T cell surface markers and markers of activation. The frequency of CD8⁺ T cells was significantly decreased in the

Figure 3.2. IL-21R signaling is required for fibrosis following bleomycin-induced lung inflammation.

- (A) Lung sections were stained with Masson's Trichrome. Magnification =20X.
- (B) Lung extracts were quantitated for collagen concentration by a colorimetric assay. Data are representative of three independent experiments (PBS→B6, n=6; PBS→*Il21r*^{-/-}, n=4; Bleo→B6, n=11; Bleo→*Il21r*^{-/-}, n=11; Bleo→*Il21*^{-/-}, n=5). Data shown are means ± SEM.
- (C) Lung sections harvested at day 14 were stained for α-smooth muscle actin to detect activated fibroblasts.
- (D) Survival of B6 and IL21R^{-/-} mice depicted following high-dose bleomycin treatment (0.15U/mouse); (B6 n=5; *Il21r*^{-/-} n=4).

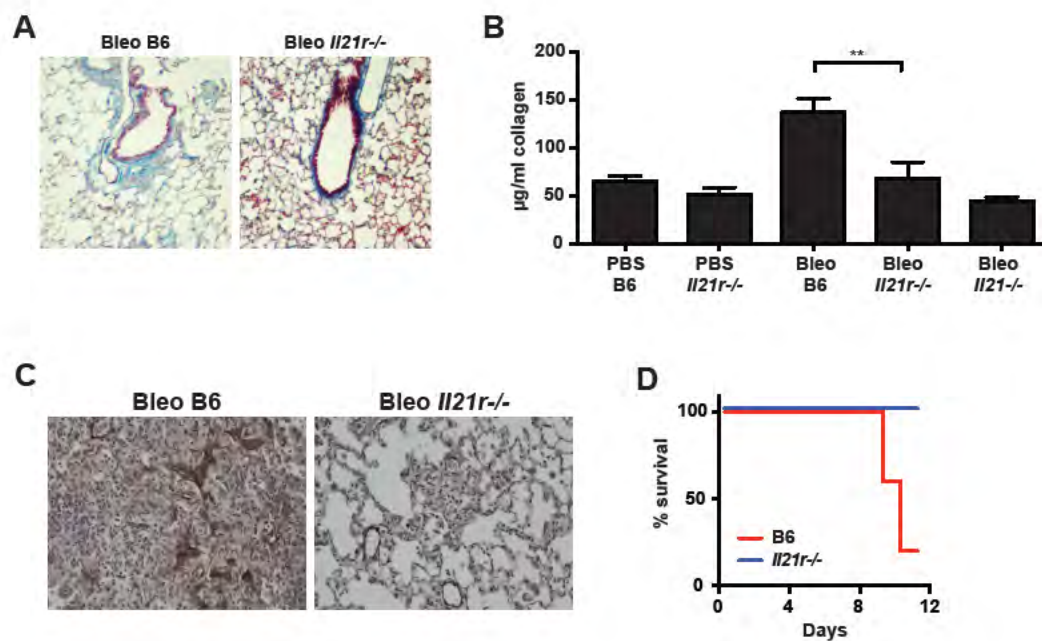


Figure 3.2. IL-21R signaling is required for fibrosis following bleomycin-induced lung inflammation.

LDLN of *Il21r*^{-/-} mice compared to B6 controls after bleomycin injury.

Furthermore, *Il21r*^{-/-} mice had roughly half (an average of 48% less than B6) the frequency and number of CD8⁺ T cells infiltrating the lung (Figure 3.3A) and LDLN (Figure 3.3B) compared to B6 mice after bleomycin injury. The total average number of CD4⁺ T cells was decreased by only 30% in *Il21r*^{-/-} mice compared to B6 controls after bleomycin treatment (Figure 3.3B). The ratio of CD4⁺ to CD8⁺ T cells was also significantly increased in bleomycin-treated *Il21r*^{-/-} mice (Figure 3.3C). However, expression of the activation marker CD69 on both CD4⁺ and CD8⁺ T cells was comparable between bleomycin-treated B6 and *Il21r*^{-/-} mice (Figure 3.3D). CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells (Tregs) can dampen inflammation, including T cell responses (Shevach, 2009). However, the frequency of Tregs in LDLN of *Il21r*^{-/-} mice was not increased relative to B6 mice, suggesting that Tregs are not likely to be responsible for decreased CD8⁺ proliferation or recruitment (Figure 3.4). These data show that IL-21 is a key factor in maximizing the CD8⁺ T cell response during lung fibrogenesis, and has a lesser effect on the CD4⁺ T cell response.

Protection from fibrosis in *Il21r*^{-/-} mice is associated with a decrease in IL-13, but not IL-17A, production by T cells.

IL-17A has been identified as a pro-fibrotic mediator in the bleomycin model (Oh et al., 2011; Wilson et al., 2010), however, it is dispensable in other models of fibrosis (Lo Re et al., 2010). Because IL-21 is an amplifier of both

Figure 3.3. IL-21/IL-21R interactions are required for optimal CD8⁺ T cell recruitment to the lung following bleomycin injury.

B6 and *Il21r*^{-/-} mice were treated with bleomycin (bleo; 0.05U/mouse) or PBS (control) i.t. and sacrificed at day 14.

(A) At day 14, LDLN cell suspensions were analyzed by flow cytometry for CD4⁺ and CD8⁺ T cell frequency.

(B) Summary of the total number of CD4⁺ and CD8⁺ T cells in LDLN at day 14.

(C) From data above, the ratio of CD4⁺ T cells to CD8⁺ T cells was determined.

(D) LDLN T cell activation at day 14 was analyzed by flow cytometry for surface expression of CD69. Data are compiled from two independent experiments with similar results. (PBS→B6, n=6; PBS→*Il21r*^{-/-}, n=4; Bleo→B6, n=8; Bleo→*Il21r*^{-/-}, n=8)

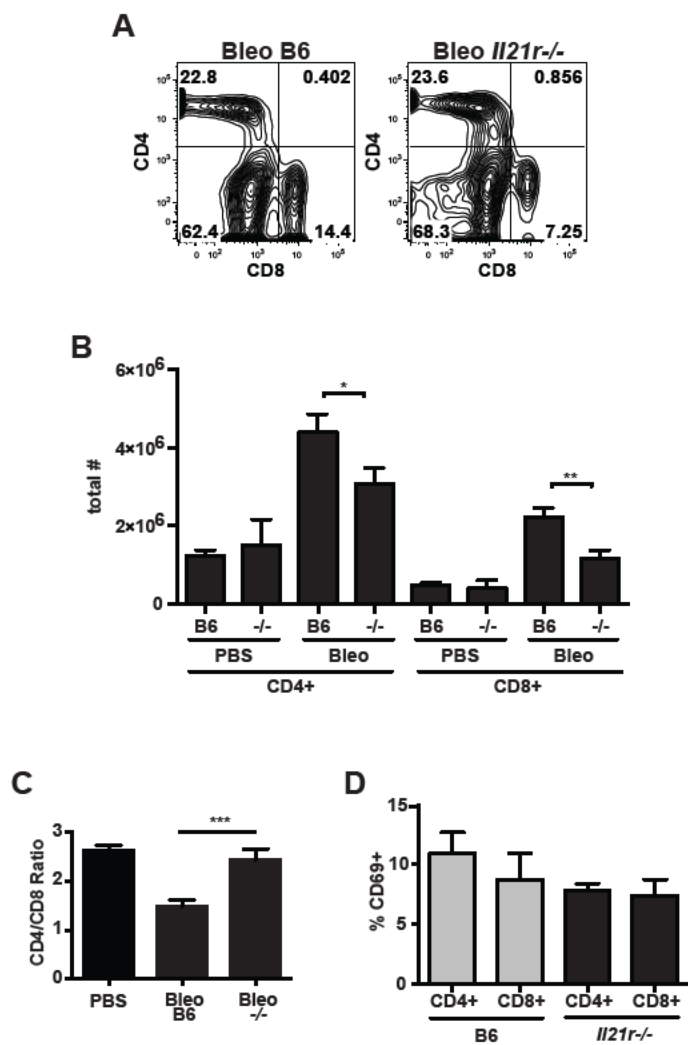


Figure 3.3. IL-21/IL-21R interactions are required for optimal CD8⁺ T cell recruitment to the lung following bleomycin injury.

Figure 3.4. Increased Treg activity is not responsible for protection from fibrosis in IL-21R deficient mice.

LDLN Treg frequency at day 14 was assessed by co-expression of CD25 and Foxp3 in the CD4⁺ gate as determined by flow cytometry. Data are compiled from two independent experiments with similar results. (PBS→B6, n=6; PBS→*Il21r*^{-/-}, n=4; Bleo→B6, n=8; Bleo→*Il21r*^{-/-}, n=8)

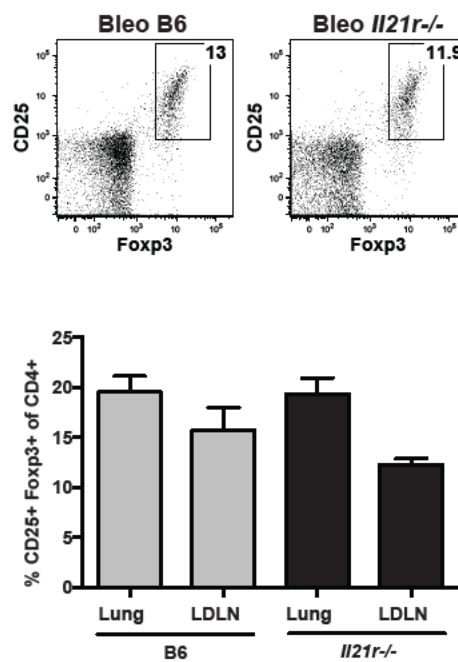


Figure 3.4. Increased Treg activity is not responsible for protection from fibrosis in IL-21R deficient mice.

Th17 and Th2 responses (Frohlich et al., 2007; Korn et al., 2007), one question that remains is to what extent IL-21 promotes Th17 or Th2 responses within a microenvironment that favors the differentiation of both. To investigate the role of IL-21 in pro-fibrotic Th2 and Th17 responses, we analyzed LDLN T cells for cytokine production. At day 14 following intratracheal bleomycin injury, single-cell suspensions of LDLN cells obtained from PBS and bleomycin treated mice were stimulated in vitro with plate-bound anti-CD3 for 24 hours and supernatants were then analyzed by ELISA for IL-17A, as a readout of Th17 cells, and IL-13, as a readout for Th2 cells. T cells from the bleomycin treated B6 and *Il21r*^{-/-} mice made comparable amounts of IL-17A (Figure 3.5A). However, T cells from bleomycin treated *Il21r*^{-/-} mice secreted three-fold less IL-13 than those from bleomycin treated B6 mice (Figure 3.5B). Surprisingly, as determined by intracellular cytokine staining of T cells from LDLN, *Il21r*^{-/-} mice had a significantly lower frequency of IL-13 producing CD8⁺ T cells (Figure 3.5C-D). The decreased frequency of IL-13 producing CD8⁺ T cells, taken together with the decreased total number of CD8⁺ T cells (Figure 3.3B) resulted in strikingly fewer IL-13 producing CD8⁺ T cells overall. These data indicate that bleomycin-induced CD8⁺ T cell differentiation into pro-fibrotic, type 2 effectors is highly dependent on IL-21. Preliminary data from human tissue confirms IL-13 upregulation in fibrotic disease (Figure 3.5E).

Figure 3.5. Lack of IL-21R significantly impairs IL-13 production in response to sterile lung injury.

(A, B) LDLN cell suspensions collected from PBS and bleo-treated mice at day 14 were stimulated with anti-CD3 and culture supernatants were harvested 24 hours later and assayed for IL-17A and IL-13 by ELISA. Data are compiled from two independent experiments with similar results. (PBS→ B6, n=6, Bleo→B6, n=9; Bleo→ *Il21r*^{-/-}, n=7).

(C, D) LDLN T cells from bleo-treated were re-stimulated and cytoplasmic staining was performed for IL-13. Data in *C* are compiled from three independent. (Bleo→B6, n=12; Bleo→ *Il21r*^{-/-}, n=10). Data in *D* are representative of three independent experiments that yielded similar results.

(E) Formalin-fixed, paraffin-embedded lung tissue sections from SSc patients and healthy controls were stained for IL-13 by immunohistochemistry. Positive IL-13 staining was observed in three SSc sections and not in any of three samples of healthy lungs.

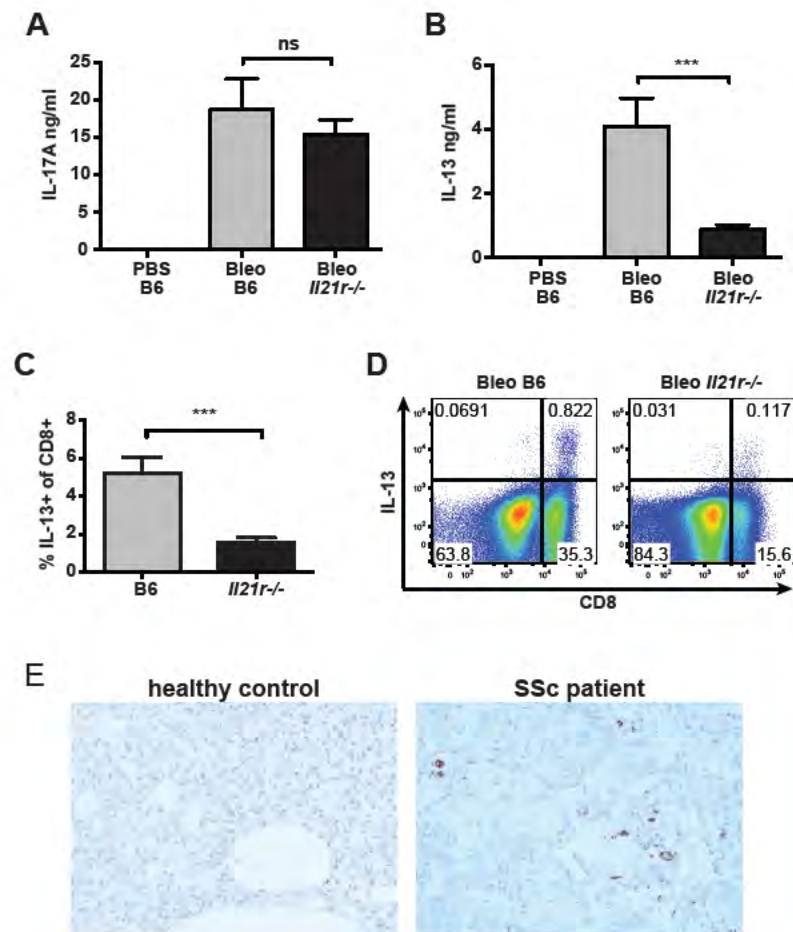


Figure 3.5. Lack of IL-21R significantly impairs IL-13 production in response to sterile lung injury.

IL-21 promotes Tc2 differentiation in vivo and in vitro

After determining that IL-21 had a role in bleomycin-induced fibrosis, we sought to determine the cellular source of IL-21. In accordance with a previous study using IL-21 reporter mice (Spolski et al., 2012), we found that a small population of CD4⁺ T cells produces IL-21 in the lung constitutively (Figure 3.6A) and these T cells preferentially express both CD44, a marker of antigen experience, and CD62L, an adhesion molecule that controls migration to lymph nodes. The frequency of IL-21⁺ CD4⁺ T cells that co-expressed CD44 and CD62L was roughly ten-fold higher than CD44/CD62L double positive CD4⁺ T cells in the total population (Figure 3.6B), suggesting that these cells could be part of a memory-like pool. To identify the source of IL-21 following lung injury, we instilled bleomycin i.t. and harvested lungs at day 7. Single-cell suspensions from lung tissue were stimulated in vitro for cytoplasmic staining. Quite unexpectedly, i.t. bleomycin induced IL-21 production from both CD4⁺ and CD8⁺ T cells in the lung (Figure 3.6C).

Since IL-21R deficiency had shown such dramatic effects on bleomycin induced fibrosis and the frequency of CD8⁺ T cells, we next asked whether IL-21 by itself could elicit fibrosis and CD8⁺ T cell production of IL-13 in lung. Cytoplasmic staining of lung tissue leukocytes from mice treated with rIL-21 showed that by day 7, a remarkably high frequency of CD8⁺ cells were producing IL-13 (Figure 3.7A). Strikingly, at day 10, mice treated with rIL-21 i.t. showed evidence of inflammation and fibrosis, which was detected histologically by H&E

Figure 3.6. IL-21 is constitutively expressed by a small population of lung CD4⁺ T cells, and is upregulated in both CD4 and CD8 T cells in response to bleomycin or IL-21 i.t.

(A) Single cell suspensions from lungs of untreated B6 and *IL21*^{-/-} mice were stimulated with PMA/ionomycin in the presence of GolgiStop for 5 hours. Intracellular IL-21 in CD4, CD8, and $\gamma\delta$ T cells was analyzed by flow cytometry after surface and cytoplasmic staining.

(B) CD44 and CD62L expression of CD4⁺ IL-21⁺ cells (the population for the gate in A) was determined by flow cytometry. For A-B, results are from two independent experiments. (B6 n=6, *IL21*^{-/-} n=2).

(C) B6 mice were treated with PBS, bleomycin (0.05U/mouse) or rmlL-21 (1 μ g/ml) i.t. On day 7, lung leukocytes were stimulated with anti-CD3 in the presence of GolgiStop and stained for intracellular IL-21. (PBS n=2, bleo n=5, IL-21 n=4).

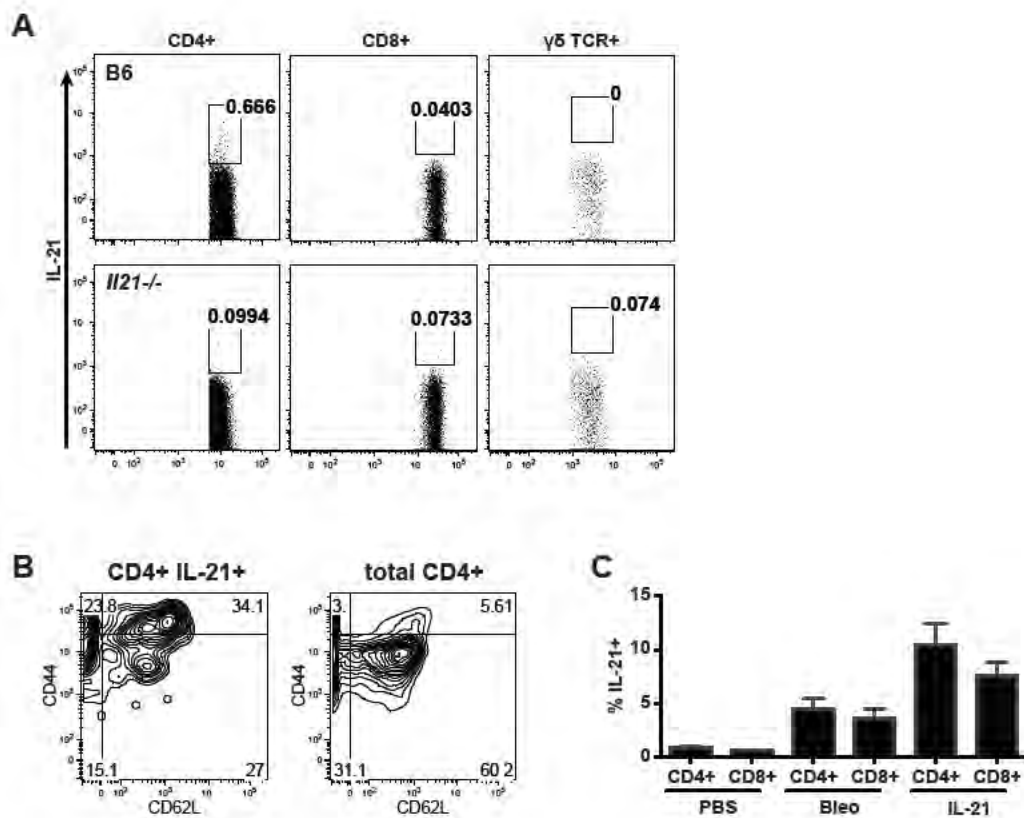


Figure 3.6. IL-21 is constitutively expressed by a small population of lung CD4⁺ T cells, and is upregulated in both CD4⁺ and CD8⁺ T cells in response to bleomycin or IL-21 i.t.

and trichrome stains (Figure 3.7B) and by quantitative collagen assay (Figure 3.7C). Thus, IL-21 drives CD8⁺ T cells to differentiate into cells that produce IL-13 and IL-21 (Tc2²¹ cells), and can drive fibrosis even in the absence of other lung injury.

The combination of IL-4 and IL-21 efficiently promotes the *in vitro* differentiation of CD8⁺ T cells into Tc2²¹ cells.

IL-21 can enhance cytokine production by Th2 cells through autocrine and paracrine mechanisms (Stritesky et al., 2011). To better understand how IL-21 promotes the differentiation of Tc2²¹ cells, we decided to evaluate the importance of IL-21 and other cytokines in the *in vitro* generation of Tc2²¹ cells. It has previously been reported that Tc2 cells require IL-2 and IL-4 for differentiation (Croft et al., 1994; Sad et al., 1995). However, IL-2 is not strongly upregulated in the lungs in response to bleomycin injury (Gur et al., 2000). Moreover, many IPF patients do not exhibit IL-2 upregulation (Homolka et al., 2003; Kitasato et al., 2004). Therefore, we sought to determine whether IL-21 was sufficient for Tc2 differentiation *in vitro* as determined by IL-13 production. We activated purified CD8⁺ T cells and assessed the ability of IL-21 to support their capacity to produce IL-13 in conjunction with IL-4, with or without IL-2. We found that IL-21 could supplant IL-2 in Tc2 differentiation, and no synergistic effect was observed by adding both IL-2 and IL-21 (Figure 3.8).

Figure 3.7. Sterile lung injury and IL-21 drives Tc2 differentiation in the lung and fibrosis.

(A) B6 mice were treated with PBS, bleomycin (0.05U/mouse) or rmIL-21 (1 μ g/ml) i.t. On day 7, lung leukocytes were stimulated with anti-CD3 in the presence of GolgiStop and stained for intracellular IL-21 and IL-13. (PBS n=2, bleo n=5, IL-21 n=4).

(B) BALB/c mice were treated with a single instillation of rmIL-21 i.t. and at day 10, lungs were harvested and formalin fixed, paraffin-embedded sections stained by H&E and Masson's Trichrome. Magnification = 20X.

(C) Collagen concentration in lung extracts from mice in (B) was measured by colorimetric assay. Bars represent means \pm SEM. In (B) and (C) Results shown are compiled from four independent experiments with three to four mice per group.

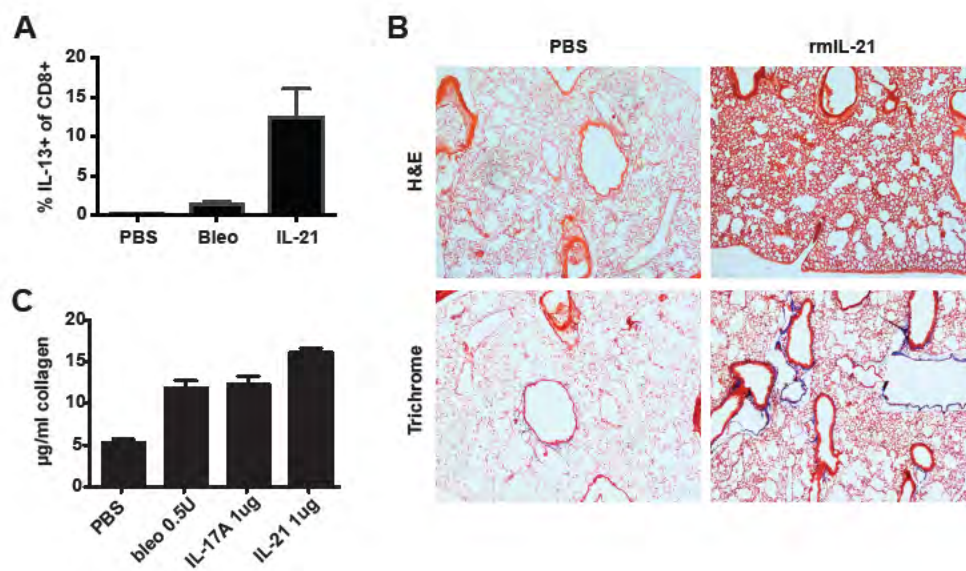


Figure 3.7. Sterile lung injury and IL-21 drives Tc2 differentiation in the lung and fibrosis.

Figure 3.8. IL-21 drives Tc2 differentiation in vitro.

Purified splenic CD8⁺ T cells from naïve B6 mice were cultured in the presence of the indicated cytokines for 4 days. The cells were then re-stimulated using anti-CD3 in the presence of GolgiStop and cytoplasmic staining for IL-13 was performed. A representative flow cytometry plot is shown. Data are representative of five independent experiments.

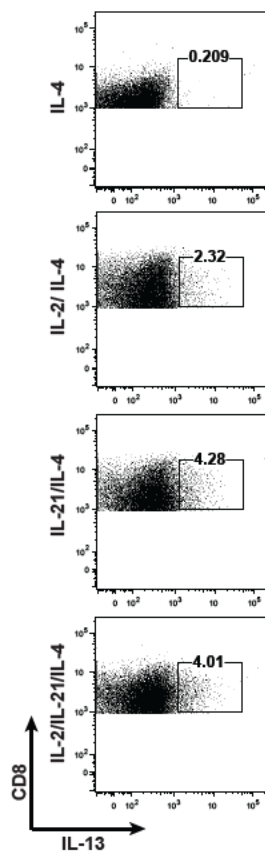


Figure 3.8. IL-21 drives Tc2 differentiation *in vitro*.

Tc2 cells self-prime with IL-21.

Since Th17 cells can be amplified by autocrine and/or paracrine IL-21 during differentiation (Wei et al., 2007), we hypothesized that autocrine IL-21 could amplify Tc2 differentiation. Using an *in vitro* activation system, we skewed wild type and IL-21-deficient purified CD8⁺ T cells using IL-2 or IL-21 with or without IL-4. Intriguingly, only IL-21/IL-4, but not IL-2/IL-4 or IL-21 alone, generated effectors capable of copious IL-21 secretion by CD8⁺ T cells upon re-stimulation as measured by ELISA (Figure 3.9A). We confirmed IL-21 production using primary splenic CD8⁺ T cell cultures from IL-21-mKate fluorescent reporter mice (Figure 3.9B). Moreover, in accordance with our hypothesis, there was a striking difference in IL-13 production by IL-21-deficient and IL-21 sufficient Tc2 cultures. IL-21-deficient CD8⁺ T cells did not secrete IL-13 when treated with IL-21 and IL-4, but wild type B6 CD8⁺ T cells secreted high levels of IL-13 (Figure 3.9C). Therefore, paracrine IL-21 drives CD8⁺ T cell secretion of IL-21, which in turn promotes IL-13 production through an autocrine pathway.

To determine whether Tc2 (IL-2 cultured) and Tc2²¹ cells had differing ability to drive fibrosis *in vivo*, we generated OT-I (ova-specific) effectors activated in the presence of IL-4, IL-4 + IL-2, or IL-4 +IL-21. We adoptively transferred activated OT-1 cells into mice expressing membrane-bound ova under the lung-specific CC10 promoter (CC10-ova mice). Injection of IL-4 + IL-2 activated OT-I Tc2 cells caused a modest increase in BAL cell number but no increase in BAL collagen. IL-4 + IL-21 activated OT-I Tc2 cells caused both an

Figure 3.9. Autocrine IL-21 promotes Tc2 phenotype.

(A) Purified splenic CD8⁺ T cells from naïve B6 mice were cultured in the presence of the indicated cytokines for 4 days. The cells were then re-stimulated using anti-CD3 without additional cytokines. Cell supernatants were harvested at 24 hours and assayed for IL-21 by ELISA. Data shown are compiled three independent experiments. Data shown are means \pm SEM.

(B) Purified splenic CD8⁺ T cells from IL-21-mKate reporter mice were cultured in the presence of the indicated cytokines for 4 days. The cells were analyzed by flow cytometry without re-stimulation. Representative of two experiments.

(C) Purified CD8⁺ T cells from WT and *Il21*^{-/-} mice were stimulated as indicated and the concentration of IL-13 in the culture supernatants was determined. Representative of three independent experiments. Data shown are means \pm SEM.

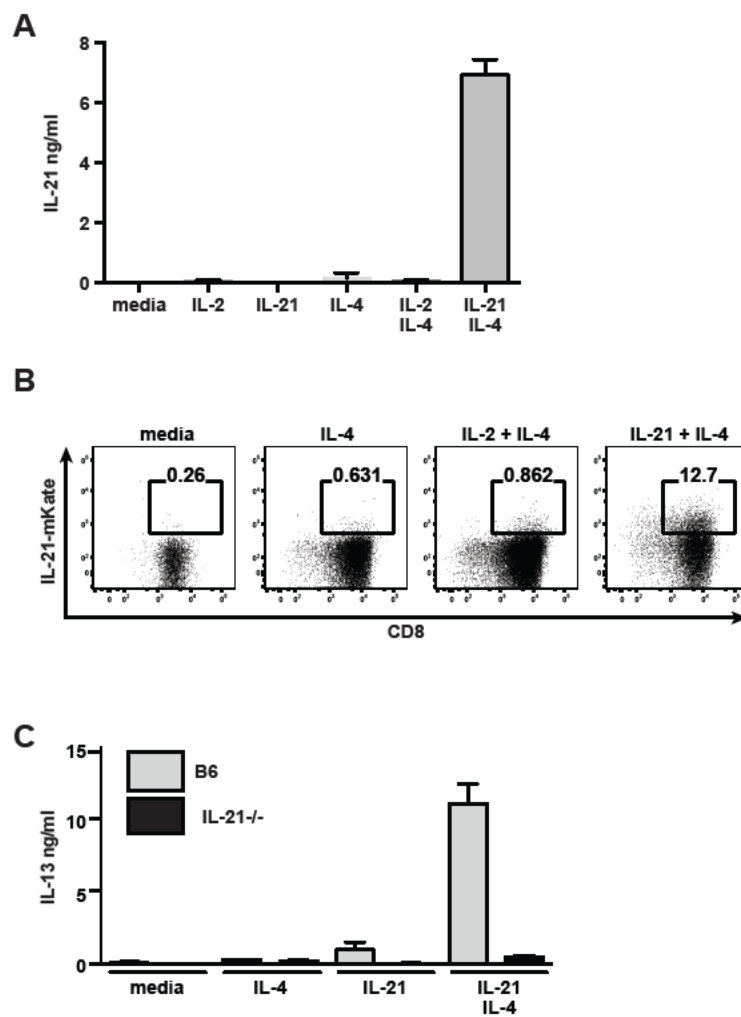


Figure 3.9. Autocrine IL-21 promotes Tc2 phenotype.

increase in BAL cell number and collagen (Figure 3.10). These data indicate that naïve T cells activated in the presence of IL-4 and IL-21 preferentially acquire pro-fibrotic activity.

CD25 signaling and TGF- β regulate Tc2 differentiation.

We next wanted to determine whether IL-2 signaling impacted Tc2 differentiation in response to IL-4 + IL-21. Quite interestingly, blockade of the high-affinity IL-2 receptor, CD25, enhanced IL-13 production almost two-fold at the expense of IFN- γ production (Figures 20A-B), suggesting that upon activation, high-affinity IL-2 signaling does not drive CD8⁺ T cells into Tc1 and Tc2 differentiation pathways evenhandedly, but rather inhibits Tc2 differentiation.

TGF- β has both potent pro-fibrotic and anti-inflammatory functions. To understand how these roles affected Tc2 differentiation, we also skewed Tc2 cells in the presence of TGF- β . As expected, TGF- β limited proliferation of CD8⁺ T cell cultures (data not shown). However, the TGF- β treated CD8⁺ T cells did produce IFN- γ , but not IL-13 (Figures 3.11A-B). Additionally, we found that TGF- β in conjunction with IL-4 decreased the level of IL-21R expression (Figure 3.11C), thereby downregulating CD8⁺ T cell responsiveness to IL-21. We again used CD8⁺ T cells purified from IL-21 reporter spleen, and found that TGF- β caused a decrease in IL-21 production (Figure 3.11D), concomitant with decreased IL-21 sensitivity (Figure 3.11C). This effect was not induced by either TGF- β or IL-4 alone. This finding suggests that while TGF- β has indispensable

Figure 3.10. Tc2²¹ cells are profibrotic in vivo.

Purified splenic CD8⁺ T cells from naïve B6 mice were cultured in the presence of the indicated cytokines with IFN- γ neutralization (30 μ g/ml) for 6 days. The cells were then re-stimulated using anti-CD3 and anti-CD28 without additional cytokine. Two days after re-stimulation, 5x10⁶ T cells were injected per mouse. BAL fluid was harvested after sacrifice and assayed for collagen 1A1 by ELISA.

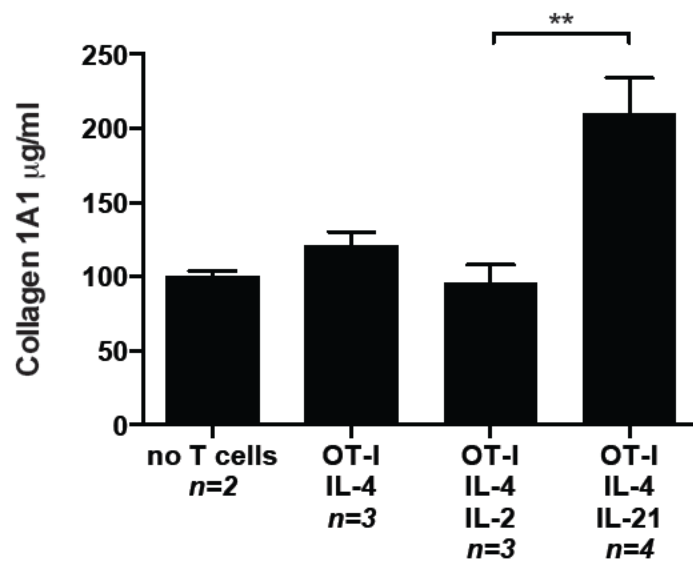


Figure 3.10. Tc2 (21) cells are profibrotic *in vivo*.

pro-fibrotic effects on stromal cells such as fibroblasts, it negatively regulates pro-fibrotic CD8⁺ T cells. Taken together, these data indicate that IL-21 can supplant IL-2 to promote Tc2 differentiation during fibrogenesis, and that high affinity IL-2R signaling and TGF- β counter-regulate the effects of IL-21 signaling.

IL-21R-sufficient CD8⁺ T cells restore fibrotic phenotype of IL-21R-deficient mice.

As shown above, CD8⁺ T cell numbers and function were most impacted by IL-21R deficiency in the *in vivo* bleomycin-induced fibrosis studies, and CD8⁺ T cells also produced large amounts of IL-13 when activated in vitro the presence of IL-21 + IL-4. Therefore we considered the possibility that IL-21-responsive CD8⁺ T cells are required for lung fibrosis in response to sterile lung injury. To test this hypothesis, purified splenic B6 CD4⁺ or CD8⁺ T cells from untreated mice were adoptively transferred into both B6 and *Il21r*^{-/-} mice and the recipients were subsequently treated with bleomycin. LDN were then collected at day 14, re-stimulated *ex vivo* with plate-bound anti-CD3, and assayed for cytokine production by ELISA. Remarkably, injection of B6 CD8⁺ T cells “rescued” defective IL-13 production by LDN T cells from bleomycin treated *Il21r*^{-/-} recipients, but had little effect on the IL-13 response of B6 recipients (Figure 3.12A). In contrast, LDNs from the B6 and *Il21r*^{-/-} recipients produced similar levels of IFN- γ (Figure 3.12B), and thus it is unlikely that the adoptively transferred T cells enhanced fibrosis by developing into tissue destructive, type 1

Figure 3.11. CD25 signaling and TGF- β regulate Tc2 differentiation *in vitro*.

(A-B) Purified splenic CD8⁺ T cells from naïve B6 mice were cultured in the presence of the indicated cytokines and antibodies for 4 days. The cells were then re-stimulated using anti-CD3 in the presence of GolgiStop and cytoplasmic staining for IL-13 and IFN- γ was performed. A representative flow cytometry plot is shown in A. Bars represent means \pm SEM. Data are representative of five independent experiments.

(C) Surface IL-21R expression on cultured CD8⁺ T cells was analyzed by flow cytometry at day 5 post-primary stimulation. Data are representative of three independent experiments.

(D) Purified splenic CD8⁺ T cells from IL-21-mKate reporter mice were cultured in the presence of the indicated cytokines for 4 days. The cells were analyzed by flow cytometry without re-stimulation. Representative of two experiments.

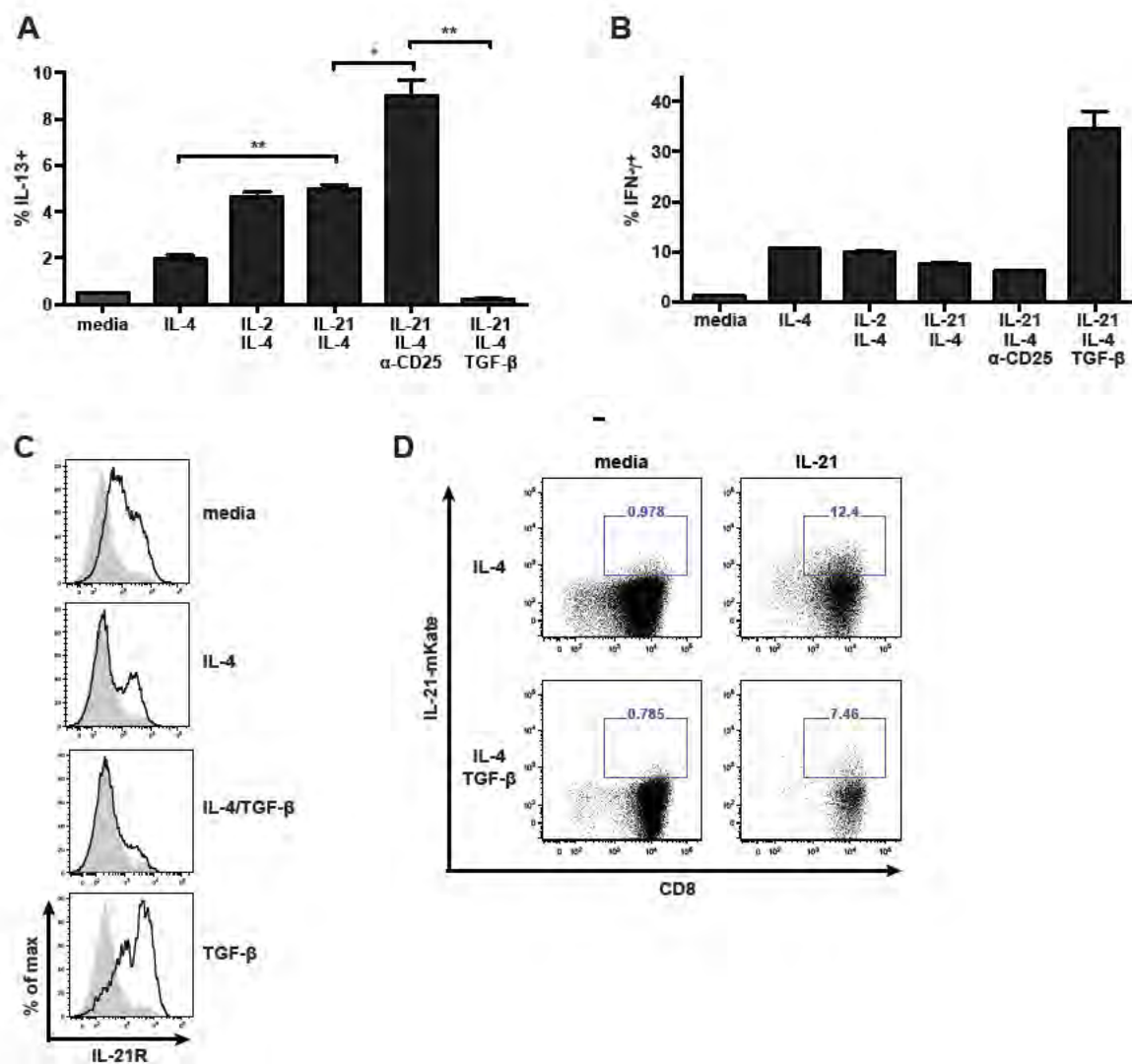


Figure 3.11. CD25 signaling and TGF- β regulate Tc2 differentiation *in vitro*.

effectors. Moreover, adoptive transfer of IL-21R-sufficient CD8⁺ T cells into *Il21r*^{-/-} mice, but not B6 mice, led to a significant increase in collagen concentration in lung extracts after bleomycin treatment. Adoptive transfer of IL-21R-sufficient CD4⁺ T cells had a negligible effect on collagen deposition (Figure 3.12C). Restoration of fibrosis and fibroblast activation by IL-21R-sufficient CD8⁺ T cells in *Il21r*^{-/-} mice treated with bleomycin was also confirmed histologically by immunohistochemical staining for collagen and α -smAc (Figure 3.12D). Thus, IL-21 responsive CD8⁺ T cells are necessary and sufficient for reconstituting the fibrotic phenotype following lung injury in *Il21r*^{-/-} mice.

The ability of wt CD8⁺ T cells, but not wt CD4⁺ T cells to restore fibrosis in *Il21r*^{-/-} mice was somewhat surprising given that Th2 cells have been closely associated with fibrosis. It seemed likely that the cytotoxic capacity of CD8⁺ T cells could render them more pathogenic than CD4⁺ T cells *in vivo*. I hypothesized that increased cytotoxicity of Th2 cells would drive fibrosis. T cells from mice deficient in the death receptor express higher levels of FasL on their surface (Chu et al., 1995), which could heighten cytotoxic potential. FasL can be cleaved by matrix metalloproteinases. The membrane bound form is pro-apoptotic and pro-inflammatory and the soluble form can be inhibitory (Hohlbaum et al., 2000). Our lab generated a transgenic mouse with a deletion in the FasL cleavage site (Δ CS), which leads to increased pro-inflammatory function of FasL (Bossaller et al., 2013). If crossed to the DO11 Tg, when injected, these cells would theoretically respond preferentially in the lung of a mouse expressing ova

Figure 3.12. IL-21R on CD8⁺ T cells is required for optimal IL-13 production and collagen deposition.

(A) 10^6 purified splenic CD4⁺ or CD8⁺ T cells from naïve B6 mice were injected i.v. into B6 and *Il21r*^{-/-} mice at day -1. At day 0, mice were treated i.t. with PBS or bleo. LDLN cell suspensions from PBS and bleo-treated mice were collected at day 14 and stimulated with anti-CD3. Cell supernatants were harvested at 24 hours and assayed for IL-13.

(B) Cells were prepared and stimulated as above and culture fluids were assayed for IFN- γ .

(C) Lungs were harvested at day 14 and lung extracts were quantitated for collagen concentration by Sircol assay.

(D) Lung sections obtained from day 14 were stained for α -smooth muscle actin to detect fibroblast activation Magnification = 20X.

Data are representative of two independent experiments (PBS→B6, n=5; PBS→*Il21r*^{-/-}, n=4; Bleomycin→B6, n=9; Bleomycin→*Il21r*^{-/-}, n=8; Bleomycin→B6 + wt CD4, n=5; Bleomycin→B6 + wt CD8, n=8; Bleomycin→*Il21r*^{-/-} + wt CD4, n=5; Bleomycin→*Il21r*^{-/-} + wt CD8, n=9). Bars represent means \pm SEM.

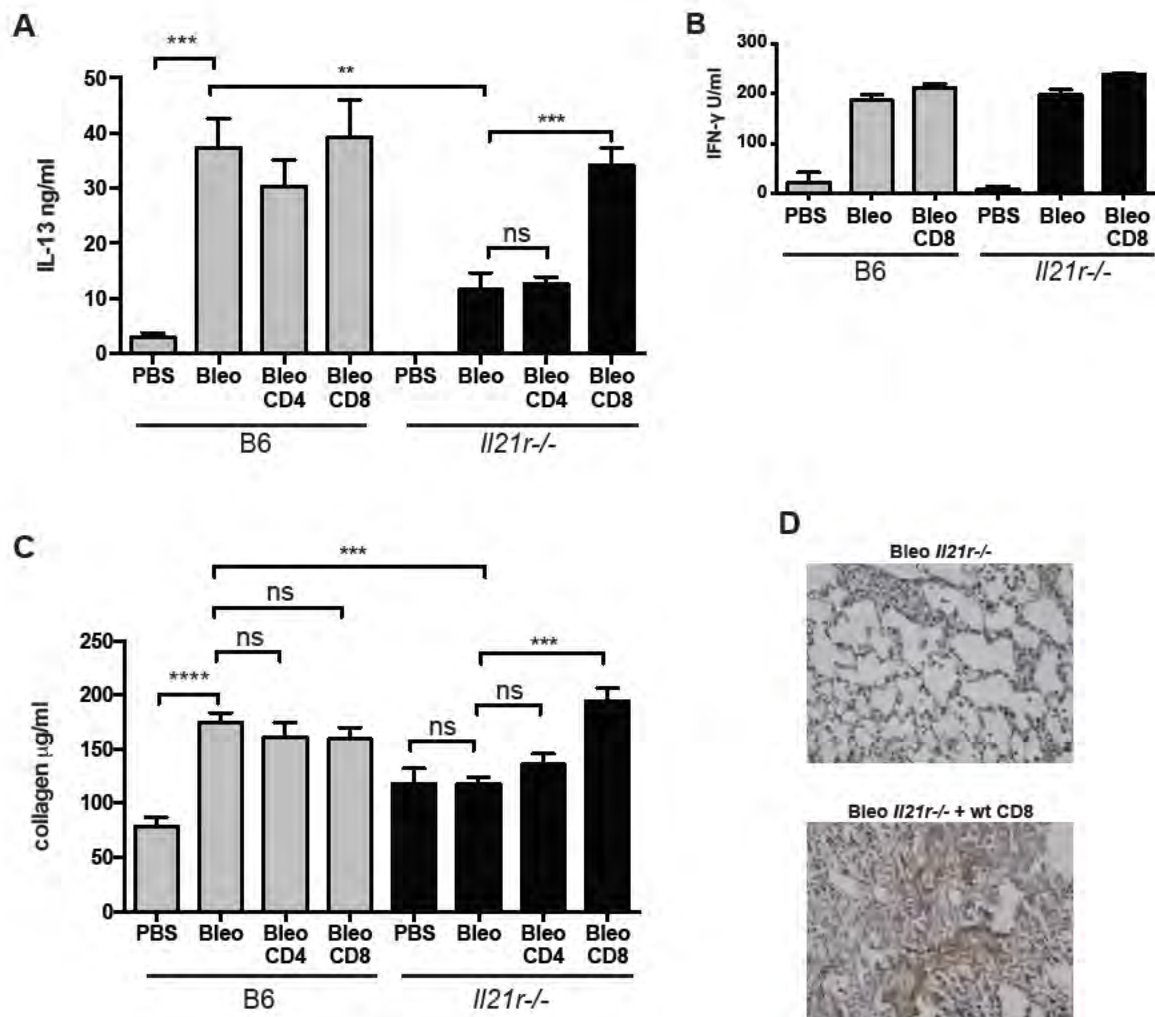


Figure 3.12. IL-21R on CD8 T cells is required for optimal IL-13 production and collagen deposition.

in the lung. To investigate the role of cytotoxicity in a system that was readily available in our lab, we made use of a double transgenic mouse line, which expresses TGO (membrane-bound OVA fusion protein) under the control of a lung-specific promoter in the presence of dox (CC10-TGO). I injected CC10-TGO mice (on dox) with Th2 cells differentiated *in vitro* with T cells from DO11, DO11-Fas^{lpr}, or DO11-Fas^{lpr}xFasL^{ΔCS/ΔCS} mice. CC10-TGO mice injected with DO11-Fas^{lpr}, or DO11-Fas^{lpr}xFasL^{ΔCS/ΔCS} cells had increased collagen deposition in the lung as measured by blinded scoring Masson's trichrome stained lung slides compared to mice that received DO11 cells with wt FasL (Figure 3.13A,C). LDLN T cells from recipients of all three genotypes of T cells secreted IL-13 in response to anti-CD3, with no significant difference between groups (Figure 3.13B). CC10-TGO mice had a higher frequency of DO11 cells in the lung compared to TGO only hosts (Figure 3.13D), and many produced IL-4 after re-stimulation (Figure 3.13E). These preliminary experiments suggest that cytotoxicity may be an important component of Tc2-driven pathology.

Discussion

Fibrosis is a frequent outcome of chronic inflammation, but despite extensive investigation, the pathways responsible for the transition from inflammation to fibrosis remain incompletely understood. Over-exuberant type 2 responses characterized by high levels of IL-4 and IL-13 are often linked to fibrogenesis, however, the initiating signals for such responses are not clear. Through the

Figure 3.13. Increased cytotoxicity of CD4⁺ T cells may promote fibrosis.

5x10⁶ Th2 cells differentiated *in vitro* with T cells from DO11, DO11-Fas^{lpr}, or DO11-Fas^{lpr}xFasL^{ΔCS/ΔCS} mice were injected into CC10-TGO or TRE-TGO mice on dox. At day 18, mice were sacrificed and lungs and LDLN were harvested. A-B show data from one experiment, C-E show data from a separate experiment.

(A) Results of blinded scoring of lung sections stained with Masson's trichrome.

(B) LDLN cell suspensions were stimulated with anti-CD3. Cell supernatants were harvested at 24 hours and assayed for IL-13.

(C) Representative lung sections stained with Masson's trichrome.

(D) Frequency of DO11 T cells in LDLN of CC10-TGO or TRE-TGO mice tallied from flow cytometry plots.

(E) Intracellular cytokine staining of ex vivo re-stimulated DO11 T cells LDLN of CC10-TGO or TRE-TGO mice.

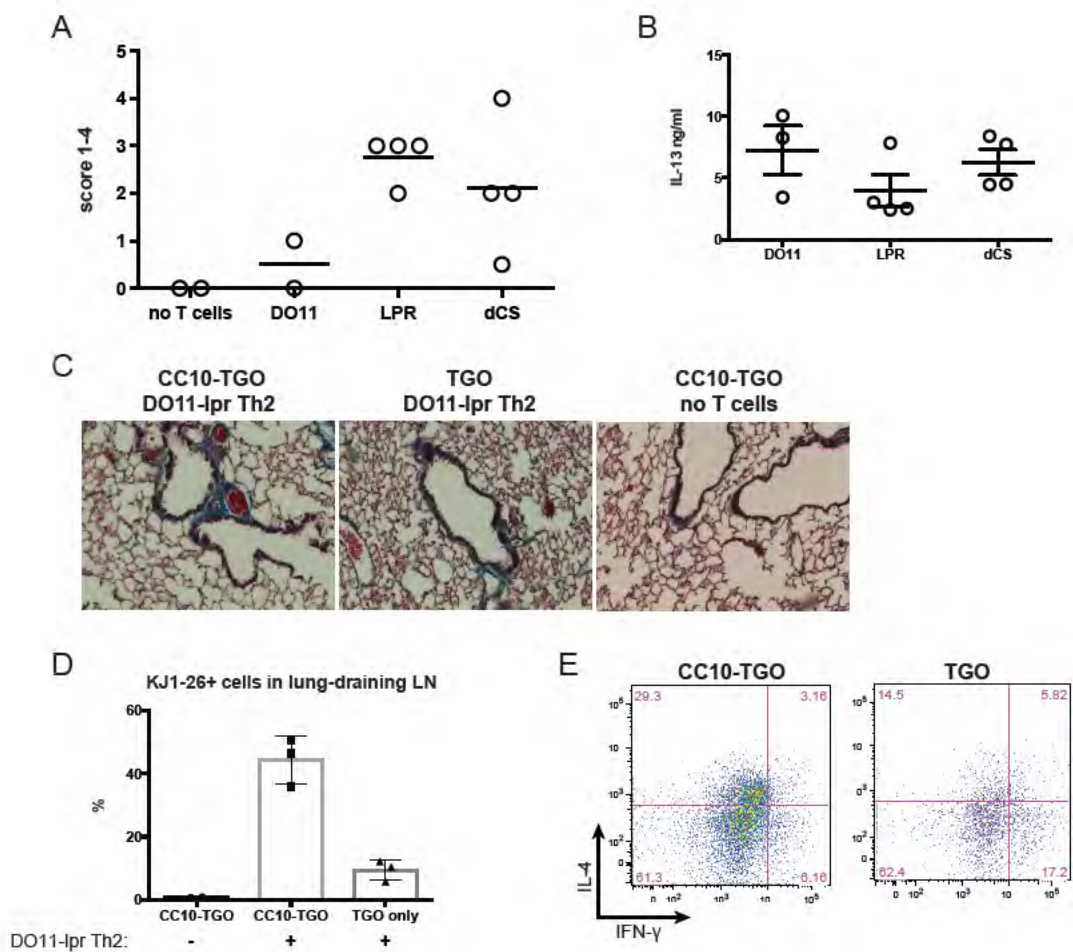


Figure 3.13. Increased cytotoxicity of CD4⁺ T cells may promote fibrosis.

analysis of IL-21R-deficient mice, we have now shown that lung fibrosis and optimal IL-13 production in response to bleomycin lung injury is dependent on IL-21R signaling, thus identifying IL-21 as a potential therapeutic target for IPF and SSc. Previous work showed that bleomycin induced lung fibrosis is ameliorated by neutralization of IL-13 or depletion of IL-13 responsive cells (Belperio et al., 2002; Jakubzick et al., 2003; Rosada et al., 2010). In accordance with these studies, we found that IL-21R deficiency and protection from fibrosis was associated with a striking reduction in IL-13 production by lung T cells *ex vivo*. Intriguingly, protection from fibrosis in *Il21r*^{-/-} mice was not associated with a significant reduction of inflammation. *Il21r*^{-/-} mice have also been shown to develop less severe fibrosis in a schistosomiasis-associated liver fibrosis model, however, this effect was attributed to IL-21 induction of Th2 cells and the promotion of increased macrophage sensitivity to Th2 cytokines such as IL-4 and IL-13 (Pesce et al., 2006). A recent study identified IL-21 as the link between G-CSF-mobilized hematopoietic stem cells and CD4⁺ T cell-independent sclerodermatous graft-versus-host disease (Hill et al., 2010). The data presented here further question whether CD4⁺ T cells are sole mediators of fibrosis. We show that in the bleomycin model, IL-13 secreting CD8⁺ T cells, or Tc2 cells, and not Th2 cells, were profoundly impacted by IL-21R deficiency. This finding was unexpected since CD4⁺ T cells have been charged with driving fibrosis through the production of both IL-17A (Oh et al., 2011; Wilson et al., 2010) and IL-13 (Belperio et al., 2002; Fichtner-Feigl et al., 2006).

IL-21 has been implicated in the induction and amplification of both Th2 (Frohlich et al., 2007; Pesce et al., 2006; Wurster et al., 2002) and Th17 cells (Korn et al., 2007), however, in the case of Th17 cells the data have been less clear-cut (Coquet et al., 2008; Sonderegger et al., 2008). In our experiments, IL-17A production was not affected by loss of IL-21R signaling. Thus, our work confirms the tissue- and context-specific role of IL-21 in Th17 differentiation. Two studies have shown an unexpected role for IL-17A derived from Th17 cells in the bleomycin model of pulmonary fibrosis (Oh et al., 2011; Wilson et al., 2010). Oh et al. injected IL-17A neutralizing antibodies at both early and later time points and found that while IL-17A was required early in the response, IL-17A blockade after day 10 did not ameliorate lung fibrosis (Oh et al., 2011). Wilson et al. also found that IL-17A was required for bleomycin induced lung fibrosis at an early time point (day 7), when IL-13 was dispensable (Wilson et al., 2010). Other studies have implicated IL-13 in pulmonary fibrosis at more commonly examined time points such as days 21-28 (Jakubzick et al., 2003; Rosada et al., 2010), as well as earlier time points (Belperio et al., 2002). In this paper we show that despite the presence of Th17 cells in the lung and LDLN, as well as comparable IL-17A production by LDLN T cells, *IL21r*^{-/-} mice were protected from bleomycin-induced fibrosis. These studies, taken together with the data presented here, suggest that IL-17A has an important role in the initial stages of fibrosis, however, IL-21-dependent IL-13 production is required for persistent fibrosis through day 21. Accordingly, Th17 cells could be an important source of the IL-

21 that skews CD8⁺ T cells toward the production of IL-13 (Tc2), in addition to the IL-21 secreting cells we found in naïve mice (Fig. 4A). Interestingly, such IL-21-dependent Th2 and Tc2 cells could suppress the further development of Th17 cells through the production of IL-4 (Cooney et al., 2011), thus reinforcing a type 2-biased environment. These data may explain why increased levels of IL-17A are detected in SSc patient sera at early, but not later stages, of disease (Radstake et al., 2009) and support a biphasic immunopathology driving fibrosis.

Our results further showed that CD8⁺ T cells, namely Tc2 cells, were most profoundly impacted by IL-21R deficiency. Previously, the physiological significance of Tc2 cells in vivo had been controversial since most functional studies of Tc2 cells used in vitro-skewed cells for adoptive transfer. In vitro-differentiated Tc2 cells are less potent effectors in pulmonary viral clearance and tumor rejection when compared to Tc1 cells (Cerwenka et al., 1999; Helmich and Dutton, 2001). However, in murine asthma, Tc2 cells develop and increase both airway inflammation and hyper-responsiveness (Miyahara et al., 2004). We now show that IL-21-dependent Tc2 cells are required for lung fibrosis after sterile lung injury, as both IL-13 production and fibrosis were restored by transferring IL-21R^{+/+} CD8⁺ T cells into *IL21r*^{-/-} mice treated with bleomycin. This result points to a novel role for IL-21 in Tc2 differentiation. Studies by other investigators have shown that Tc2 differentiation can be achieved in vitro using a combination of IL-2 and IL-4 (Croft et al., 1994; Sad et al., 1995). Here we have shown that IL-21, in conjunction with IL-4, can replace the need for exogenous IL-2 in Tc2

differentiation, a new and unanticipated role for IL-21 in CD8⁺ T cell responses. However, the role of IL-21 was not neutral, since, CD25 blockade enhanced Tc2 differentiation in response to IL-21/IL-4, which again pointed to a specific role for IL-21 during fibrogenesis that cannot be replaced by IL-2.

Interestingly, our *in vitro* experiments also showed that upon activation, CD8⁺ T cells secrete IL-21 at low-levels to amplify IFN- γ production through an autocrine process. Higher levels of IL-21 (detectable by ELISA) were secreted only in response to both IL-4 and IL-21, but not to either alone. Furthermore, a population of CD8⁺ T cells recruited to the lung after instillation of bleomycin or IL-21 produced IL-13. Our finding that CD8⁺ T cells produce IL-21 *in vivo* was unexpected, and could reflect a phenotype that is specific to the sterile type 2 environment and not mounted during viral or parasitic infections. To the best of our knowledge, this is the first report of IL-21 production by CD8⁺ T cells with relevance to a disease model. IL-21 message was detected in T-bet and Eomesodermin double-knockout Tc17 cells, however, IL-21 was not detected in Tc17 cultures by ELISA (Ciric et al., 2009). We now show that CD8⁺ T cell production of IL-21 is induced in the type 2 environment, and that autocrine/paracrine IL-21 production promotes Tc2 responses. Interestingly, fibroblasts in the gut express IL-21R and upregulate matrix metalloproteinases in response to IL-21 (Monteleone et al., 2006), raising the possibility that CD8⁺ T cell-derived IL-21 could act directly on fibroblasts. Moreover, IL-21 production by Tc2 cells could further reinforce a type II immune response due to the robust

effects of IL-21 on CD4⁺ T cells and innate immune effectors (Rochman et al., 2009; Wan et al., 2013). Strikingly, IL-21 instillation into the lungs of mice in the absence of other injurious stimuli led to fibrosis in addition to the development of both IL-13 and IL-21 producing CD8⁺ T cells. Thus, the foregoing data show that IL-21 is both necessary and sufficient for pulmonary fibrosis.

In studying the differentiation of Tc2 cells, we found that TGF- β , a cytokine abundantly expressed in fibrotic tissues, specifically inhibited Tc2 differentiation. The finding that TGF- β decreased IL-21R expression on CD8⁺ T cells when combined with IL-4 was surprising due to the pro-fibrotic roles of both cytokines. However, this could reflect a “brake” on fibrogenesis by decreasing positive feedback through the IL-21R. It should be noted that bleomycin-induced lung fibrosis is a self-limiting disease after single intratracheal instillations, and a TGF- β -dependent feedback mechanism may be responsible for the spontaneous resolution of fibrosis. TGF- β is also produced by macrophages (Song et al., 2000), including alternatively activated macrophages, which are known to accumulate in the fibrotic lung (Gibbons et al., 2011; Pechkovsky et al., 2010). The capacity of macrophage-derived TGF- β to suppress Tc2 differentiation might explain why macrophage depletion after fibrosis is established was found to exacerbate collagen deposition (Duffield et al., 2005). Additionally, the decrease in the expression of the IL-21R in response to TGF- β could be a mechanism by which Treg suppression of Tc2 cells is promoted. Since IL-21 impairs Treg function (Attridge et al., 2012; Petrelli et al., 2011; Schmitz et al., 2013), Treg-

derived TGF- β could thereby limit an IL-21R/IL-21 positive feedback loop, thus ensuring the potency of nearby Tregs by dampening local IL-21 production. As IPF has certain features of autoimmunity such as clonal T cell expansion and autoantibodies (Feghali-Bostwick et al., 2007), Treg robustness could impact disease progression.

In addition to a role for IL-21 in Tc2 skewing, we found that IL-21R signaling was required for optimal CD8⁺ T cell proliferation/recruitment. Both the frequency and total number of CD8⁺ T cells were reduced in *Il21r*^{-/-} mice during fibrogenesis compared to wild type controls. The frequency and number of CD4⁺ T cells was affected to a lesser extent. This data is consistent with a significant body of work characterizing mitogenic effects of IL-21 on CD8⁺ T cells. Furthermore, IL-21 is critical for controlling chronic viral infection, as it is a component of CD4⁺ T cells help to CD8⁺ T cells (Elsaesser et al., 2009; Yi et al., 2009), and is also critical for production of antibody (Ozaki et al., 2002). In the NOD model of diabetes, lack of IL-21R signaling also decreases CD8⁺ T cells and attenuates disease, however, in the NOD system, it has been reported that IL-21R⁺ CD4⁺ T cells and dendritic cells recruit cytotoxic CD8⁺ T cells into pancreatic islets (Van Belle et al., 2012). However, we found that direct engagement of IL-21R on CD8⁺ T cells was required for fibrosis and optimal IL-13 production during lung fibrogenesis. Since fibrotic disease is a Th2/Tc2 driven response, rather than the Th1/Tc1-driven NOD disease, our results could point to a requirement for IL-21R for a robust CD8⁺ T cell effector activity that is

specific to the type 2 environment caused by sterile fibrotic lung injury. Whether our findings could be extended to other sterile injury models of fibrosis such as silica, or an infection model such as hypersensitivity pneumonitis remains to be determined.

CD8⁺ T cells have been positively correlated with disease severity in patients with lung fibrosis (Atamas et al., 1999) and CD8⁺ T cell activation is increased in early, diffuse SSc (Radstake et al., 2009). We now establish a role for CD8⁺ T cells among the repair/fibrosis class of effectors during sterile inflammation. Importantly, a number of studies have linked Tc2 cells to fibrosis in human patients. CD8⁺ T cells from systemic sclerosis patients secrete copious IL-13 and can activate fibroblasts in an IL-13 and STAT-6-dependent manner (Fuschiotti et al., 2013). Additionally, in the peripheral blood of systemic sclerosis patients, a higher frequency of CD8⁺ T cells produces IL-13 than CD4⁺ T cells (Fuschiotti et al., 2013; Fuschiotti et al., 2009). It has also been reported that the frequency of Tc2 cells in the lungs of IPF patients is correlated with disease severity and shortness of breath (Atamas et al., 1999), and CD8⁺ T cells are as, if not more, abundant than CD4⁺ T cells in the lungs of IPF patients (Daniil et al., 2005; Kradin et al., 1986; Papiris et al., 2005). These studies, taken together with the current report, challenge the predominant CD4⁺ T cell-centric view of fibrosis. Our data clearly point to an IL-21/IL-13 axis during fibrogenesis, requiring CD8⁺ T cells as effectors on both sides of this axis. Overall, these results have important implications for the rational design of IL-21 targeted

therapies that could be used to treat fibrosis resulting from conditions such as SSc, IPF, or chronic inflammation.

Experimental Procedures

Mouse Strains

BALB/c and C57BL/6 mice were purchased from the Jackson Laboratory. IL-21R knockout mice originally made by Warren Leonard (Ozaki et al., 2002) were provided by Oliver Dienz (University of Vermont) and IL-21 knockout mice were obtained from Lexicon- Mutant Mouse Regional Resource Centers and generously provided by Dr. S. Swain (University of Massachusetts Medical School). All mouse experiments were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Lung treatments

12-16 week old mice were anesthetized by isoflurane inhalation. 50µl of sterile bleomycin sulfate (Sigma, 0.05U/mouse (low dose) or 0.15U/mouse (high dose) dissolved in sterile PBS), recombinant mouse IL-21 (R&D Systems, 1µg/mouse dissolved in sterile PBS), or PBS were instilled into the lungs of mice by oropharyngeal aspiration (Walters and Kleeberger, 2008). For experiments where mice of different genotypes were compared, the mice were age-matched within two weeks difference in date of birth.

Lung and lymph node tissue harvesting

Mice were sacrificed by cervical dislocation and immediate removal of the diaphragm. Lungs were perfused with PBS through the right ventricle to remove blood. Bronchoalveolar lavage fluid (BALF) was collected with three flushes of a single aliquot of 0.5ml PBS. Lung draining lymph nodes were dissected and dissociated in Hank's buffered saline solution (HBSS) using frosted glass slides. The right bronchus was sutured and the right and postcaval lobes were removed and placed in HBSS, minced, and forced through a 100µm nylon mesh filter to generate a single-cell suspension. The left lobe was inflated with 10% neutral buffered formalin, the left bronchus was sutured, and the left lobe was then removed and submerged in 10% buffered formalin at room temperature until processed for paraffin embedding (>24 hours).

Collagen Measurement

Where the left lung lobe was not used for histology, the entire lobe was minced into fine pieces and placed in 2 ml of a solution of acetic acid (Sigma, 0.5M) and pepsin. After 48 hours, the solution was spun at 4C for 20 minutes, and these lung extracts were frozen until collagen concentration assay. Sircol (Biocolor, Belfast, UK) or Sirius Red Total Collagen Detection kit (Chondrex) were used according to the manufacturers instructions with similar results.

Histopathology and immunohistochemistry

Formalin fixed, paraffin embedded lung tissue was sectioned into 5 μ m sections and stained by hematoxylin and eosin or Masson's trichrome stain.

Immunohistochemistry for α -smooth muscle actin (Santa Cruz Biotechnology, clone 1A4, 1:500 dilution) was performed without antigen retrieval, goat anti-mouse IgG2a-HRP (Santa Cruz Biotechnology, 1:500 dilution) was used as a secondary antibody, with diaminobenzene (DAB; Vector Laboratories) as a substrate. Mayer's hematoxylin was used as a counterstain for immunohistochemistry.

Flow cytometry

Single cell suspensions were surface or intracellular stained with combinations the following antibodies: IL-21R-biotin, CD3-PE-Cy7, -biotin (clone 145-2C11), CD4-eFluor450, -APC (clone RM4-5), CD8-FITC, -APC, -PE-Cy7 (clone 53-6.7), CD25-PerCP-Cy5.5 (clone PC61.5), CD69-eFluor450, - PerCP-Cy5.5, IL-21-APC (clone FFA21), IL-13-PE (clone eBio13A), IL-17A-PerCP-Cy5.5 (clone eBio17B7), Foxp3-PE (clone FJK-16s) all from eBioscience. Streptavidin-PerCP was from BD Biosciences.

For Foxp3 staining, cells were fixed and permeabilized using the Foxp3 Transcription Factor staining buffer kit (eBioscience) according to the manufacturers instructions. For intracellular cytokine staining, single-cell suspensions were stimulated ex-vivo with plate-bound anti-CD3 (BioLegend, 5

µg/ml) or PMA (Sigma, 100ng/ml) plus ionomycin (Sigma, 1µg/ml) in complete RPMI for 2 hours at 37°C. GolgiStop (BD Biosciences, 1:100) was added and cultures were incubated for an additional 2-3 hours at 37°C.

Cytokine measurements

Lung draining lymph node single-cell suspensions (200,000-400,000 cells/well) were seeded onto anti-CD3 (BioLegend; 5µg/ml) coated plates in RPMI. Supernatants were collected at 24 hours and analyzed by ELISA for IL-13 (BD Biosciences), IL-21 (eBioscience), IFN-γ (BD Biosciences), and IL-17A (eBioscience).

***In vitro* T cell differentiation**

CD8⁺ T cells were purified from spleen cell suspensions by positive selection (BD iMag CD8α beads). Cells were stimulated with plate bound anti-CD3 (2 µg/ml) and soluble anti-CD28 (BioLegend, 2 µg/ml) with or without recombinant mouse IL-4 (BD Biosciences, 10 ng/ml), recombinant human IL-2 (BD Biosciences, 50 ng/ml), recombinant mouse IL-21 (R&D Systems, 50ng/ml), or recombinant human TGF-β (Peprotech, 5ng/ml). Anti-CD25 antibody was purified from PC61 hybridoma supernatant (a generous gift from Dr. M. Shlomchik, University of Pittsburgh) and used at a concentration of 20 µg/ml. Cells were split on day 2 and then as needed. On day 4 or 5, T cells were re-stimulated with anti-CD3 for

intracellular cytokine staining or re-stimulated to harvest supernatants for ELISA after 24 hours.

Statistical methods

Data analysis was performed using Prism (GraphPad) software. p values were calculated using unpaired two-tailed Student's t test. In all figures, ns (not significant) denotes a $p > 0.05$, *, $p \leq 0.05$, **, $p \leq 0.01$, ***, $p \leq 0.001$. Error bars represent standard error of the mean (SEM).

Chapter IV: Discussion

An initial goal of the li-TGO model of SLE was to induce disease with activated DO11 T cells, and then mimic lupus flares by turning TGO expression on and off to study the biology of B cells during simulated “flares.” The ability to turn TGO on and off would theoretically allow for monitoring of the kinetics and magnitude of the ANA response, which may provide evidence for whether memory autoreactive B cells are involved in flares. A previous graduate student, Karen Lee, showed that ANA+ li-TGO mice 6 weeks post DO11 Th2 adoptive transfer, withdrawal of dox led to a decrease in ANA titers. This suggests that OVA-specific effector cells, most likely DO11 Th2, maintained autoreactive B cell production of autoantibodies through cognate interactions. This result is somewhat counterintuitive. Using a traditional view of antibody responses, ANA-producing B cells should have eventually become plasma cells and stopped requiring T cell help. This result points to at least two possibilities: (1) autoreactive antibody secreting cells do not become true plasma cells and require T cell help for survival and/or (2) DO11 Th2 drive a differentiation pathway that is halfway between anergic and plasma cell and these cells still have a shorter half life than a non-autoreactive cell. That is, DO11 cells persist when antigen persists, and when DO11 cells persist, they can provide help to new anergic cells that develop into short-lived plasmablasts. Short-lived plasmablasts such as these have been identified in anti-RF B cell transgenic mice on an MRL/lpr background (William et al., 2005), where they participate in

an anti-RF response. The dependence on TGO expression, which I interpret as dependence on DO11 cell persistence, exhibited in Th2→li-TGO lupus like disease could be specific to early stages of disease. It is likely that as disease progressed, epitope spreading would occur, and autoreactive T cells specific for true auto-Ags could engage in cognate interactions with autoreactive B cells, thus precluding a requirement for DO11 T cells. In this scenario, we would expect that an enduring break in B cell tolerance had occurred. I attempted to address this question by generating mice that expressed TGO on half of their B cells, and did not express TGO on the other half in order to compare antibody production by each population following DO11 Th2 adoptive transfer. I generated BM chimeras that were a 50/50 mix of li-TGO BM and C.B-17 BM. C.B-17 cells provide a source of normal, non-TGO expressing B cells, but also express *Ighb*, allowing for detection of antibody secreted by these cells using anti-allotype secondary reagents. Curiously, less than 10% of B cells detected in reconstituted li-TGO/C.B-17 mixed BM chimeras expressed IgM[b] on their surface, suggesting that C.B-17 B cells survived poorly or that C.B-17 BM engrafted poorly. However, this experiment could be difficult to interpret given that nonspecific T cell help could promote autoantibody production by C.B-17 B cells, especially in the context of inflammation.

The nature of li-TGO B cell/DO11 T cell interactions is currently unclear. DO11 T cells account for less than 1% of CD4⁺ T cells in the spleen several weeks or months after adoptive transfer, however, as described above, TGO

expression must be maintained for ANA titers to remain high. To definitively show whether productive li-TGO B cell/DO11 T cell interactions occur, li-TGO B cells and CFSE-labeled DO11 T cells would need to be transferred into an MHC-II-deficient host to determine whether T cell divisions are B cell-dependent. Whether li-TGO B cell/DO11 T cell interactions are sufficient for ANA production is presumed, but also unclear, and could be addressed using a long-term version of a similar experiment.

The low frequency of adoptively transferred DO11 cells raises several questions about their fate. Autoantibody responses can occur in germinal centers or at extrafollicular sites, and a determination of whether DO11 Th2 become Tfh, Tefh, or localize with B cells in lymph nodes and spleen is important in the context of T-B interactions in lupus. Tfh development and participation in germinal centers requires cognate and ICOS/ICOS ligand interactions with B cells (Tangye et al., 2013). As discussed above, nonspecific T cell help can enhance autoantibody production. However, this scenario may not be optimal or even likely to occur in an autoimmune individual; it is possible that Ag-specific T-B interactions would outcompete nonspecific interactions, especially during early phases of disease.

A variety of genetic and environmental factors are thought to lead to SLE (Choi et al., 2012). Crossing an autoreactive BCR Tg mouse line to an autoimmune prone strain can promote evasion of B cell tolerance by autoreactive

B cells (Mandik-Nayak et al., 1999; Santulli-Marotto et al., 2001; Wang and Shlomchik, 1999). It is therefore likely that the barriers to ANA production that we find in the li-TGO model would be less. For example, I would predict that on an *lpr* or *gld* background, li-TGO B cells would become spontaneously activated by immune complexes and could then activate naïve DO11 T cells in a dox-dependent manner. A more sophisticated version of this experiment would utilize mixed bone marrow chimeras, with half of the bone marrow being li-TGO x *Ighb*, and the other half being BALB/c-*lpr-gld* (to prevent FasL-mediated graft versus-host-disease or susceptibility to Fas-mediated death) or normal BALB/c. This would allow for assessment of ANA production by li-TGO B cells in an autoimmune environment, without requiring li-TGO B cells to be genetically autoimmune prone.

LNSCs have a clear role in peripheral tolerance through both autoreactive CD8 T cell deletion and suppression of CD4 and CD8 T cell proliferation (Fletcher et al., 2010; Fletcher et al., 2011; Khan et al., 2011; Lukacs-Kornek et al., 2011; Siegert et al., 2011). Suppression of T cell proliferation by LNSCs is mediated by iNOS (Lukacs-Kornek et al., 2011), which may also induce Tregs (Niedbala et al., 2007). A role for iNOS in regulating Treg trafficking has not been shown, however LNSCs express other factors, such as IL-7, which could impact Treg phenotype. Literature on stroma and Tregs is extremely limited – and this potential interaction may have been overlooked due to the relatively low level of MHC-II on LNSCs. However, MHC-II is increased on LNSCs during

inflammation, and Tregs may respond to low levels of MHC-II due to higher affinity for self-peptide. It would be quite interesting to know whether LNSCs from an inflamed environment (e.g. an autoimmune mouse lymph node) would interact with Tregs differently, but this would likely be dependent on the mediators of stromal/Treg interactions. The first step in identifying the molecule(s) responsible for CD62L upregulation on Tregs would be to determine whether it is a soluble factor or a membrane-bound ligand. In addition to IL-7, PD-L1 could be a likely target, since it is both expressed by LNSC (Reynoso et al., 2009) and can promote Treg induction and influence function (Francisco et al., 2009).

The circumstantial evidence for activation and preferential lymph node homing in the prevention of ANA production in this system is clear, however, the true functional capacity of CD62L⁺ Tregs has not been shown in our model. These cells are very protective in graft-versus-host disease responses, and are feasible to adoptively transfer (Ermann et al., 2005; Fu et al., 2004; Szanya et al., 2002). The suppressive function of CD62L⁺ DO11 Tregs on a per-cell basis could be tested in vitro, since some or all of these cells could express other molecules that make them more potent suppressors than CD62L⁻ Tregs. To determine whether CD62L⁺ Tregs exhibit enhanced homing to sites of autoreactive B cell activation, CD62L⁺ Tregs from DO11 x li-TGO or wild type mice could be transferred into an autoimmune model with a similar genetic background such as BALB/c-lpr. I hypothesize that localization in lymph nodes,

rather than increased suppressive capacity, would attenuate autoreactive B cell activity.

While it is presumably conventional Tregs that prevent autoantibody production in li-TGO chimeras with host TGO expression, other regulatory populations could be responsible. Tfh cells promote B cell antibody production in germinal centers through a variety of membrane-bound and soluble factors including CD40/CD40L interactions and IL-21 (Tellier and Nutt, 2013). Tfh that express Foxp3 have been described, and may have a regulatory role in germinal centers (Chung et al., 2011; Linterman et al., 2011). Autoimmune responses that lead to the production of autoantibodies can occur in extrafollicular foci rather than germinal centers (Odegard et al., 2008). Whether Tefh can also express Foxp3, and whether the self-Ag/rAPC-dependent regulatory population that may be responsible for attenuated B cell activation and ANAs in li-TGO→li-TGO chimeras is a regulatory Tfh/Tefh rather than a conventional Treg remains to be determined.

In the li-TGO system, we used Th2 cells to drive SLE-like disease, however, SLE has been associated with type I IFN and IFN- γ , and diseases such as SSc have been associated with deregulated Th2 responses. While we were developing a model of T cell-driven fibrosis, data on the role of Th17 cells in fibrosis shifted the Th2-bias paradigm. I hypothesized that Th17 and Th2 responses could be linked in fibrosis, and that IL-21 overproduction could lead to deregulated activity of both. We tested this hypothesis using the bleomycin

model of lung injury, which causes inflammation followed by fibrosis. We expected that the number of inflammatory cells infiltrating the lung and lung draining lymph nodes after bleomycin treatment would be dramatically reduced in IL-21R-deficient mice. Although overall cell numbers were similar between B6 and *Il21r*^{-/-} mice, the ratio of CD4⁺/CD8⁺ T cells was significantly increased in *Il21r*^{-/-} mice. The fact that IL-21R-deficiency had a greater impact on CD8⁺ T cell numbers suggests that IL-21 actively promotes CD8⁺ T cell responses, as is suggested by the increased number of CD8⁺ memory T cells in IL-21-overexpressing mice (Allard et al., 2007). *Il21r*^{-/-} mice were also completely protected from fibrosis, and this was accompanied by decreased IL-13 production by LDLN T cells re-stimulated *ex vivo*. Unexpectedly, intracellular cytokine staining revealed a decreased frequency of IL-13⁺ CD8⁺ T cells, rather than a decrease in IL-13⁺ CD4⁺ T cells. Data from patients with SSc demonstrates that type 2-skewed CD8⁺ T cells are increased in SSc patients, secrete IL-13 at levels that can activate fibroblasts (Fuschiotti et al., 2013; Fuschiotti et al., 2009), and are associated with lung function decline (Atamas et al., 1999). We have begun staining human lung tissue from healthy individuals, SSc patients, and IPF patients. We have found IL-13⁺ cells in the lungs of patients with SSc, but not healthy controls. It is of interest to determine whether any of the IL-13⁺ cells are CD8⁺ T cells, and whether they co-express IL-21.

Our data showed an unexpected autocrine role for IL-21 in promoting Tc2 differentiation. In response to the combination of IL-21 and IL-4, CD8⁺ T cells

secreted IL-21 and IL-13. IL-21 deficient cells did not make IL-13, suggesting that autocrine/paracrine feedback could be required for proper Tc2²¹ differentiation and function. This idea could be confirmed by treated CD8⁺ T cells under Tc2²¹ conditions with increasing concentrations of IL-21. We could next ask whether Tc2²¹ cells become IL-21 independent in later stages of differentiation. Transfer of Tc2²¹ cells, but not conventional Tc2 cells, caused lung fibrosis. While this could be a direct effect of IL-21, which can drive fibrosis, it could also be indicative of increased survival of Tc2²¹ cells due to their ability to secrete copious amounts of IL-21. These experiments should be repeated using a congenic marker such as Thy1.1, so that adoptively transferred cells can be detected by flow cytometry, and the numbers of conventional Tc2 versus Tc2²¹ cells can be compared. It will also be important to assess the stability of both lineages by production of IL-13 compared to other T lineage cytokines.

Our data show that IL-21R sufficient CD8⁺ T cells are required for bleomycin-induced fibrosis, but IL-21R-sufficient CD4⁺ T cells are dispensable. This result was surprising, since Th2 cells are classically associated with fibrosis. Apoptosis is involved in the pathogenesis of IPF and the bleomycin model (Thannickal and Horowitz, 2006) and perforin-deficient mice develop attenuated pulmonary fibrosis (Miyazaki et al., 2004). Taking the role of apoptosis into consideration, it is possible that while CD4⁺ T cells may secrete pro-fibrotic cytokines, the low cytotoxic potential of Th2 cells makes them ineffective. In CC10-TGO hosts injected with activated DO11 Th2, lpr cells caused more

collagen deposition, which could be a result of increased FasL on the surface of DO11 lpr cells that causes cell death and promotes fibrogenesis. One future direction could be to skew Th1, Th2, and Th17 DO11 lpr cells to compare their fibrogenic potential in CC10-TGO hosts. IFN- γ (Fielding et al., 2014), IL-13 (Wynn, 2011), and IL-17A (Oh et al., 2011; Wilson et al., 2010) have all been associated with fibrosis, and the role T cell mediated cytotoxicity in this context remains unclear.

CD8-mediated cytotoxicity would presumably involve peptide:MHC-I recognition. It is unknown whether T cells recognize self-Ag in the bleomycin model, or whether non-specific activation occurs. We are in the process of answering this question by reconstituting *Rag1*^{-/-} hosts with wild type CD8⁺ T cells or OT-I-*Rag1*^{-/-} T cells, with or without CD4⁺ T cells, then treating with bleomycin. Adoptive transfer experiments showed that OT-I Tc2²¹ cells cause increased collagen deposition in the lungs of CC10-mOVA mice. However, Tg-negative littermates did not exhibit increased collagen in BAL fluid. Data from this experiment suggests that Ag recognition by Tc2 is required for fibrosis. Because the OT-I Tc2 cells were transferred into a non-inflammatory environment, the requirement for Ag recognition could have been greater since there was no ongoing inflammation to activate CD8⁺ “bystanders.” The extent of CD8⁺ T cell contribution to pulmonary fibrosis is obscure, but the novel pathway to type 2 effector CD8⁺ T cells described here may provide a foundation for future

studies using more definitive approaches such as conditional deletion of IL-21 or IL-21R.

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